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Development of Natural Killer Cells Regulated by T-Box Transcription Factors

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Abstract
Natural killer (NK) cells play critical roles in host defense against tumors and intracellular pathogens. Various transcription factors have been reported to alter NK cell development, but the definitive transcriptional program governing this process is not well understood. Our laboratory has shown previously that the T-box transcription factors T-bet and Eomesodermin (Eomes) direct fate and function in cytotoxic T lymphocytes. We thus endeavored to define the roles of T-bet and Eomes in the differentiation of NK cells. We find that NK progenitor cells give rise to immature-phenotype NK cells, which express the death ligand TRAIL and the transcription factor T-bet but lack a combinatorial repertoire of activating and inhibitory Ly49 molecules, responsible for the broad specificity of NK cells against microbial components and missing self. T-bet appears essential to stabilize this immature lineage. Immature NK cells give rise to mature NK cells that express the integrin CD49b ($DX5^+$) and a diverse repertoire of Ly49 family receptors. Mature NK cells maintain expression of T-bet and induce expression of Eomes. T-bet is dispensable for the development of mature NK cells. Eomes, however, is critical for the development of mature NK cells. Eomes also maintains aspects of the mature phenotype. The effects of T-bet deletion and Eomes deletion are complementary and additive, as no NK cells are observed in mice lacking both T-bet and Eomes. The biological basis for stepwise maturation of NK cells appears to rest in a strict prohibition against induction of Eomes until post-natal life. Restriction of Eomes expression by fetal and adult liver limits NK development to a T-bet-dependent, immature state, whereas medullary hematopoiesis is permissive for Eomes induction and progression to NK cell maturity. These findings reveal critical, genetically separable checkpoints in maturation of NK cells.

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ABSTRACT

DEVELOPMENT OF NATURAL KILLER CELLS REGULATED BY T-BOX TRANSCRIPTION FACTORS

Scott Michael Gordon

Steven L. Reiner

Natural killer (NK) cells play critical roles in host defense against tumors and intracellular pathogens. Various transcription factors have been reported to alter NK cell development, but the definitive transcriptional program governing this process is not well understood. Our laboratory has shown previously that the T-box transcription factors T-bet and Eomesodermin (Eomes) direct fate and function in cytotoxic T lymphocytes. We thus endeavored to define the roles of T-bet and Eomes in the differentiation of NK cells. We find that NK progenitor cells give rise to immature-phenotype NK cells, which express the death ligand TRAIL and the transcription factor T-bet but lack a combinatorial repertoire of activating and inhibitory Ly49 molecules, responsible for the broad specificity of NK cells against microbial components and missing self. T-bet appears essential to stabilize this immature lineage. Immature NK cells give rise to mature NK cells that express the integrin CD49b ($DX5^+$) and a diverse repertoire of Ly49 family receptors. Mature NK cells maintain expression of T-bet and induce expression

of Eomes. T-bet is dispensable for the development of mature NK cells. Eomes, however, is critical for the development of mature NK cells. Eomes also maintains aspects of the mature phenotype. The effects of T-bet deletion and Eomes deletion are complementary and additive, as no NK cells are observed in mice lacking both T-bet and Eomes. The biological basis for stepwise maturation of NK cells appears to rest in a strict prohibition against induction of Eomes until post-natal life. Restriction of Eomes expression by fetal and adult liver limits NK development to a T-bet-dependent, immature state, whereas medullary hematopoiesis is permissive for Eomes induction and progression to NK cell maturity. These findings reveal critical, genetically separable checkpoints in maturation of NK cells.

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INTRODUCTION

Function of Natural Killer Cells

Natural Killer (NK) cells were first identified as lymphocytes with natural reactivity to tumor cell lines *in vitro* (Kiessling et al., 1975a; 1975b). Able to enact rapid, contact-dependent cytolysis without prior exposure to target cells, NK cells were immediately distinguished from $CD8^+$ cytotoxic T lymphocytes. Additionally, NK cells developed in the absence of the thymus, a primary lymphoid organ required for T cell development. T and B lymphocytes, which comprise the adaptive immune system, rearrange the antigen receptor locus in a random fashion to achieve fine antigen specificity. In contrast, NK cells came to be defined as innate lymphocytes that rely on germline-encoded, non-rearranged receptors to detect targets and to activate cytolysis and cytokine production (Lanier, 2008; Vivier et al., 2011).

The earliest interrogations into the function of NK cells led to the formulation of the “missing self hypothesis” (Kärre et al., 1986). This hypothesis referred to another key distinction between adaptive cytotoxic T cells and innate NK cells. $CD8^+$ T cells require interaction between the T cell receptor and cognate antigen presented by Major Histocompatibility Complex Class I (MHC Class I), to recognize target cells. Killer T cells become blind to potential targets when target cells downregulate MHC Class I, a strategy employed by several viruses and tumors to evade the adaptive immune response. Using implantable tumor lines

that either expressed host MHC, *i.e.* “self”, or lacked expression of self-MHC, Karre and colleagues found that tumors missing self appeared to exhibit enhanced susceptibility to natural killing (Kärre et al., 1986). The study, however, could not rule out the possibility that the MHC Class I-deficient tumor not only lacked self but expressed an activating ligand for an as-yet-undefined NK cell receptor. Later studies more specifically assayed the role of MHC deficiency on the activation of NK cells by employing a mouse genetically deficient in the β2m protein, critical for transporting Class I MHC to the cell surface (Bix et al., 1991; Liao et al., 1991; Zijlstra et al., 1989). It was observed that MHC-sufficient NK cells would reject haploidentical hematopoietic cells from the $\beta 2m^{-/-}$ host *in vitro* and *in vivo* (Bix et al., 1991; Liao et al., 1991). Further support for the missing self hypothesis came from investigations of hybrid resistance, a phenomenon in which the offspring of MHC-disparate parents would reject a bone marrow transplant from either parent (Kumar et al., 1997). Hybrid resistance was demonstrated in mice lacking adaptive immune lymphocytes, suggesting that this phenomenon was mediated by NK cells (Murphy et al., 1987). Studies of the mechanism of NK-mediated hybrid resistance employed an MHC Class I transgene to formally test the role of self-MHC. Mice expressing D^b Class I MHC rejected bone marrow from mice expressing D^d Class I MHC (Ohlén et al., 1989). Marrow from D^b mice expressing a D^d transgene was spared from rejection in D^d mice and D^b x D^d hybrids. Additionally, D^b mice transgenic for D^d rejected D^b marrow (Ohlén et al., 1989). Altogether, these data extended the missing self hypothesis and

suggested that hematopoietic cells were susceptible to NK-mediated killing if they lacked a full complement of self-MHC.

The missing self hypothesis necessarily implied that NK cells could recognize, and were somehow inhibited by, Class I MHC. But it failed to explain why NK cells from MHC Class I-deficient mice exhibited diminished killing activity *in vitro* and did not reject a bone marrow transplant from allogeneic mice (Liao et al., 1991). The missing self hypothesis predicts that $\beta 2m^{-/-}$ mice would be not be deficient in NK cell activity. Rather, MHC Class I-deficient mice should be riddled with hyperactive NK cells and NK-mediated autoimmunity. Another shortcoming of the missing self hypothesis lies in the fact that hybrid progeny reject parental hematopoietic cells but accept parental solid organ transplants (Kumar et al., 1997). This observation suggested that NK activation was not controlled entirely by inhibitory signaling by self-MHC and that other, likely activating, stimuli played a key role in NK cell activation.

Indeed, it has been appreciated that NK cells express a diverse array of activating and inhibitory receptors (Orr and Lanier, 2010; Vivier et al., 2011). In the setting of target recognition, it is the net signals transmitted from activating and inhibitory receptors that determine NK cell activation. Why, then, in the absence of inhibitory (self-MHC) signals in MHC Class I-deficient mice, do activating signals not predominate and result in widespread NK activation? In addition to the original observations in $\beta 2m^{-/-}$ mice, a battery of later studies all

pointed to the same phenomenon: similar to adaptive immune lymphocytes, NK cells appear to be “educated”, or tolerized to self (Orr and Lanier, 2010; Raulet and Vance, 2006; Joncker and Raulet, 2008; Vivier et al., 2011).

Two prevailing, though partially opposing, models are believed to explain the phenomenon of NK cell tolerance. The first, known as the “arming and disarming model” puts forth that NK cells are functionally “armed” by interactions between inhibitory receptors and cognate self-MHC (Raulet and Vance, 2006). Functional “disarming” takes place when activating receptors are chronically stimulated in the absence of balancing inhibitory receptor signaling. In other words, over-stimulated NK cells can become functionally “anergic”. The earliest evidence for the arming and disarming model came from work challenging wild-type/ $\beta 2m^{-/-}$ mixed bone marrow chimeras with bone marrow allografts. Whereas intact MHC-sufficient hosts would fully reject an MHC-deficient allograft, mixed chimeras generated on a wild-type background exhibited enhanced tolerance to MHC-deficient allografts (Wu and Raulet, 1997). Mixed chimeras generated on a $\beta 2m^{-/-}$ background accepted MHC-deficient allografts to an even greater degree. Thus, the presence of $\beta 2m^{-/-}$ cells seemed to dominantly induce tolerance in wild-type NK cells that would otherwise lyse an MHC-deficient allograft (Wu and Raulet, 1997). These data were consistent with the disarming model, in which repeated activating stimulation rendered NK cells hyporesponsive to further stimuli. Additionally, it was observed that, in wild-type animals, NK cells can be found that lack any known inhibitory receptors but express activating receptors

(Fernandez et al., 2005). Further support for the disarming model came from studies in which mice constitutively expressing activating NK ligands fostered hypoactive NK cells (Tripathy et al., 2008).

A variant of the arming model, the “licensing model” describes a process by which NK cells become functionally competent only after engaging an inhibitory receptor with MHC Class I (Elliott and Yokoyama, 2011). Evidence in support of licensing came from studying various inhibitory-receptor-expressing NK cells in allogeneic mice. It was found that only NK cells bearing an inhibitory receptor that bound the MHC haplotype of the host were functionally competent upon stimulation (Kim et al., 2005).

In mice, members of the Ly49 family of C-type lectin-like receptors recognize classical Class I MHC molecules (Hanke et al., 1999). Ly49 receptors may be inhibitory or activating, depending on the character of the intracellular tail of the receptor. Ly49A, Ly49C, Ly49G2, and Ly49I are examples of Ly49 family members that transduce signals through the immunoreceptor tyrosine-based inhibititory motif (ITIM) upon binding MHC Class I molecules (Lanier, 2008; Orr and Lanier, 2010; Vivier et al., 2011). The inhibitory Ly49 receptors are those implicated in the arming and licensing models (Elliott and Yokoyama, 2011; Joncker and Raulet, 2008). The activating Ly49 family member Ly49D transduces signals through an immunoreceptor tyrosine-based activating motif (ITAM) in response to cells expressing MHC Class I (Hanke et al., 1999). Ly49H,

another activating Ly49 receptor, binds an MHC Class I-like molecule, m157 (Arase et al., 2002). Known as an MHC decoy, m157 is encoded by murine cytomegalovirus (MCMV) and sent to the cell surface, so that the infected cell may evade the host $CD8^+$ T cell response. The m157 protein stimulates NK cells, however, and it was observed that expression of Ly49H by NK cells was required to maintain resistance to MCMV infection (Lee et al., 2001). Although additional NK receptors may play a secondary role in clearance of MCMV (Lodoen et al., 2003; Zafirova et al., 2009), mice lacking Ly49H-expressing NK cells cannot control early infection with MCMV (Lee et al., 2001).

Expressed by human NK (hNK) cells, the Ig superfamily members killer immunoglobulin-like receptors (KIRs) appear to be the functional homolog of murine Ly49 receptors. KIRs bind human leukocyte antigens (HLA), the equivalent of murine MHC, and transduce signals through intracellular ITIMs or ITAMs (Lanier, 2008). An array of other receptors decorates the surface of murine and hNK cells, and many receptors are structurally and functionally homologous between the two species. Major examples of orthologous NK receptors in mice and humans include the C-type lectin-like receptors NKG2A and NKG2D and the natural cytotoxicity receptor NKp46. The heterodimeric NKG2A/CD94 is an inhibitory receptor that recognizes the non-classical self-MHC molecule $Qa\text{-}1^b$ (Vance et al., 1998), believed to protect cells from lysis by NK cells expressing sufficient levels of NKG2A (Jia et al., 2000). NKG2A-$Qa\text{-}1^b$ interactions may also play a key role in self-tolerance of neonatal NK cells

(Sivakumar et al., 1999), nearly devoid of activating and inhibitory Ly49 receptors (Kubota et al., 1999). As opposed to NKG2A, NKG2D ligates several different molecules on the surface of target cells and transmits activating signals to the NK cell. The common thread among NKG2D ligands is that they are all self-proteins upregulated by “stressed” cells, such as tumors (Diefenbach et al., 2000; Raulet, 2003). Of note, even though tumors may express a full complement of otherwise protective MHC Class I molecules, induction of NKG2D ligands dominantly renders these stressed cells susceptible to NK-mediated lysis (Cerwenka et al., 2001; Diefenbach et al., 2001). NKp46 is an activating receptor that was first cloned on hNK cells (Pessino et al., 1998). NKp46 can activate NK cells after binding to viral hemagglutinins, typically encoded by influenza and parainfluenza viruses (Mandelboim et al., 2001).

The studies of the landscape of NK cell surface receptors and the investigations into the nature of NK cell activation and inhibition suggest: 1) that armed/licensed NK cells are educated to self-ligands and thus reject targets missing self, 2) that upregulation of stressed self-ligands allows NK cells to break tolerance to self, and 3) that NK cells can directly recognize viral components and lyse infected cells. Altogether, NK cells could be expected play critical roles in the control of certain viruses and tumors in humans. Much of the evidence for the importance of NK cells in human immunity derives from studies in pediatric patients with NK cell abnormalities, such as functional impairment or outright deficiency (Lee et al., 2007; Orange, 2006). A common theme in these subjects is increased

susceptibility to herpes viruses, including Herpes Simplex Virus, Cytomegalovirus, Epstein Barr Virus, Varicella Zoster Virus, and Papilloma Virus. These observations are consistent with the requirement for murine NK cells in MCMV infection. While deficiency of hNK cells has a clear association with susceptibility to certain viruses, and while recognition of MCMV was found to be mediated directly by Ly49H, the mechanism by which hNK cells recognize and confer resistance to human herpesviruses is not yet known.

Beyond controlling herpesviruses, hNK cells have been implicated in the response against several other infectious agents, including Hepatitis C Virus (HCV) and the Human Immunodeficiency Virus (HIV). The data to support these claims stem from association studies of HCV- or HIV-infected individuals. One such study reported that individuals homozygous for a combination of the NK receptor KIR2DL3 and the HLA molecule HLA-C1 were significantly protected from HCV disease progression, relative to heterozygous individuals or those not expressing any KIR2DL3/HLA-C1 (Khakoo et al., 2004). Expression of HLA-C1 by HCV-infected cells is advantageous due to the weak interaction between HLA-C1 and KIR2DL3. NK cells responding to HCV infection, thus, are relatively uninhibited to lyse the infected host cell. Other recent studies have shed light on the role of NK cells in the control of HIV infection. Combined expression of KIR3DS1 and HLA-B Bw4-80I was associated with reduced progression to AIDS, and enhanced reactivity of KIR3DS1^{+} hNK cells against HIV-infected, HLA-B Bw4-80I^{+} target cells was demonstrated subsequently *in vitro* (Martin et al., 2002;

Alter et al., 2007). Further investigation revealed that NK cells appear to exert a selective pressure on HIV, resulting in the evolution of viral clones resistant to NK-mediated killing (Alter et al., 2011). Specifically, polymorphisms in HIV proteins were found in KIR2DL2^{+} individuals that enhance binding of this inhibitory KIR to HIV-infected CD4^{+} T cells. In turn, enhanced binding of KIR2DL2 to its HLA ligand on target cells results in reduced NK activation and impaired lysis of infected targets.

In addition to playing a key role in antiviral immunity, NK cells also have known roles in human antitumor immunity. One such role revolves around the ability of NK cells to reject hematopoietic cells missing self. BM transplantation has been used commonly for the treatment of leukemia. The major side effect of allogeneic BM transplantation is graft-versus-host disease, in which donor-derived adaptive lymphocytes mount an immune response against recipient tissue. By the same token, donor cytotoxic T cells also mediate a graft-versus-leukemia effect, resulting in the elimination of host leukemic cells. Investigations of humanized mouse models and of human association studies revealed that donor NK cells were key players in the graft-versus-leukemia phenomenon, rejecting host leukemic cells missing donor HLA molecules (Leung et al., 2004; Ruggeri et al., 2002). Further, carefully considered KIR-mismatching appeared to be associated with improved outcomes in AML, including a substantial reduction in the rate of relapse.

Development and Homeostasis of NK Cells

As innate lymphocytes, NK cells become functionally competent during development, which provides the host with cells primed to fight infection or cancer. Like all lymphocytes, NK cells are of hematopoietic origin, so they necessarily derive from the hematopoietic stem cell. By culturing purified populations of human hematopoietic progenitors in limiting dilutions, hNK cells were found to more immediately derive from a multipotent precursor with T, B, DC, and NK potential (Galy et al., 1995). Similarly, adoptively transferred common lymphoid progenitors (CLPs) in the the mouse gave rise to T, B, and NK cells and could give rise to DCs under certain conditions (Kondo et al., 1997). In various mouse models of CLP deficiency several groups noted that T and B lymphopoiesis were markedly impaired (He and Malek, 1996; Waskow et al., 2002). The size of the NK compartment in these mice, however, was unaffected (He and Malek, 1996; Waskow and Rodewald, 2002). These data suggest that NK cells may be borne from a precursor other than the CLP, though the mouse models used may not reflect an absolute deficiency of CLPs.

Most proximally, the bone marrow-resident natural killer progenitor (NKP) generates NK cells (Rosmaraki et al., 2001). Phenotypically, NKPs are identified in the marrow as non-granular/non-lytic cells lacking classical markers of the T, B, NK, and myeloid lineages but expressing CD122, the beta chain of the interleukin-2 (IL-2) and interleukin-15 (IL-15) receptor complex. NKPs lack the potential to give rise to T or B cells and are restricted to the NK lineage (Carotta

et al., 2011; Rosmaraki et al., 2001). NKPs are believed to give rise to immature-phenotype NK cells, which express the NK1.1 antigen and NKp46 and are heterogeneous for CD27 (Chiossone et al., 2009; Kim et al., 2002; Rosmaraki et al., 2001; Walzer et al., 2007). Immature-phenotype NK cells may express some Ly49 family members and exhibit some lytic properties, including expression of the cytolytic effector molecules perforin and granzyme B (Kim et al., 2002; Rosmaraki et al., 2001). Expression of integrin α_v and the death ligand TRAIL in the steady state uniquely mark immature NK cells (Kim et al., 2002; Takeda et al., 2005). Mature-phenotype NK cells, in contrast, lack expression of integrin α_v and TRAIL and are instead marked by DX5, the antibody raised against the integrin CD49b. $DX5^+$ NK cells are highly proliferative in the steady state and express an unrestricted Ly49 repertoire. Once $DX5^+$, NK cells can further differentiate and upregulate the integrin CD11b. Terminal differentiation of $DX5^+CD11b^{high}$ NK cells occurs with induction of leukosialin (CD43) and KLRG-1 and downregulation of CD27 (Chiossone et al., 2009; Kim et al., 2002; Robbins et al., 2004).

The surface molecule common to all stages of NK cell development is CD122. Required for IL-15-responsiveness, the presence of CD122 on NK lineage cells from the NKP onward underscores the importance of IL-15 in the development and maintenance of NK cells. Indeed, among IL-2, IL-4, IL-7, and IL-15, all cytokines that signal through the common-gamma signaling unit, only IL-15 was shown to have a significant effect on NK cell number beyond the NKP

(Vosshenrich et al., 2005). In the setting of systemic absence of IL-15 or without expression on non-NK cells of IL-15Rα, which presents IL-15 in *trans* to the CD122/common-gamma complex (Dubois et al., 2002; Lodolce et al., 2001), NK cell homeostasis is severely impaired (Kennedy et al., 2000; Prlic et al., 2003). Partial rescue of NK cell development or homeostasis was noted with expression of IL-15Rα on dendritic cells alone (Castillo et al., 2009). These data implicate accessory immune and non-immune cells in the presentation of IL-15 to NK cells.

Transcription Factors in Differentiation and Maturation of NK cells

Compared to cytokine deficiencies, transcription factor deficiencies have manifested more varied and informative developmental, phenotypic, and functional phenotypes in the NK cell compartment. The earliest, most severe, and most specific impact on NK cell development was reported after deletion of the basic leucine zipper transcription factor E4BP4 (Nfil3). Consistent with the induction of *E4bp4* from the NKP stage to the immature-phenotype stage, the number of NKPs were not affected in the absence of E4BP4 (Gascoyne et al., 2009). Development past the NKP, however, was abrogated, as nearly no NK cells were found in any organs of *E4bp4*$^{-/-}$ mice (Gascoyne et al., 2009; Kamizono et al., 2009). This relative phenocopy of mice deficient in IL-15 or IL-15Rα prompted the authors to test whether E4BP4 might effect IL-15 signaling. *E4bp4* was induced in immature- and mature-phenotype NK cells after exposure to IL-15 (Gascoyne et al., 2009). Also, forced expression of E4BP4 in bone marrow progenitor cells led to partial rescue of NK cell development in *Il15ra*$^{-/-}$

mice. E4BP4 likely mediates aspects of NK development other than integration of IL-15 signals, though, as evidenced by the reduction in *Id2* and *Gata3* expression in E4BP4-deficient NK cells (Gascoyne et al., 2009).

Similar to NK cell development in the absence of E4BP4, NK development was substantially impaired in the absence of Id2 (Boos et al., 2007; Yokota et al., 1999). The DNA-binding protein inhibitor Id2 canonically inhibits basic helix-loop-helix transcription factors, such as E2A proteins, which are required for B cell development (Bain et al., 1994; Sun et al., 1991; Zhuang et al., 1994). Id2 expression typifies true NKPs, devoid of T and B lineage potential (Boos et al., 2007; Carotta et al., 2011). Unlike E4BP4-deficient mice, Id2-deficient mice appeared to exhibit some development of immature-phenotype NK cells (Boos et al., 2007). Additionally, loss of Id2 resulted in ablation of another lineage of innate lymphocytes, lymphoid tissue-inducer cells (LTi) (Boos et al., 2007; Yokota et al., 1999). The incomplete defect in NK development may reflect some functional redundancy with another Id protein or with the DNA-binding factor TOX, the absence of which was also associated with a severe deficit in NK cells and LTis (Aliahmad et al., 2010). Expression of *Id2* was reduced in the absence of TOX, and forced expression of Id2 was unable to rescue NK cell development from $Tox^{-/-}$ progenitors. These data are consistent with a model in which TOX is activated together with, or immediately downstream of, Id2 and reinforces the activity of Id2.

Several other transcription factors have been shown to alter the phenotype, functional capacity, and number of NK cells. For instance, deletion of the Ets protein family member Ets-1 resulted in a reduction in the number of NK cells (Barton et al., 1998). *Ets1*$^{-/-}$ NK cells lysed target cells poorly *in vitro* and *in vivo* and produced decreased amounts of interferon-gamma (IFN-γ) in response to stimulation *in vitro*. Loss of another Ets protein, MEF, also resulted in a decreased number of NK cells (Lacorazza et al., 2002). MEF-deficient NK cells also exhibited impaired production of IFN-γ and inefficiently lysed target cells *in vitro*. The loss of lytic ability in the absence of MEF was attributed to direct regulation of the *Prf1* gene, encoding the cytotoxic effector protein perforin, by MEF (Lacorazza et al., 2002). Deficiency of a third member of the Ets family of transcription factors, PU.1, also was found to reduce the absolute number of NK cells (Colucci et al., 2001). PU.1 is required for expression of Ly49A and Ly49D. PU.1 is dispensable, however, for NK cytotoxicity. Finally, PU.1-deficient NK cells lack expression of the alpha chain of the interleukin-7 receptor (IL7Rα) (Colucci et al., 2001).

Expression of IL7Rα was found to be a hallmark of cells derived from a GATA-3-dependent, thymic pathway of NK cell differentiation (Vosshenrich et al., 2006). GATA-3 additionally appears to direct homing of NK cells to the liver (Samson et al., 2003). And in contrast to its role in antagonizing production of IFN-γ in T cells (Ferber et al., 1999; Lee et al., 2000; Ouyang et al., 1998; Usui et al., 2003; Yagi et al., 2010), GATA-3 is essential for production of IFN-γ in NK cells. This

severe defect in IFN-γ production by *Gata3*$^{-/-}$ NK cells manifested *in vivo* with Listeriosis early after infection with *Listeria monocytogenes* (Samson et al., 2003), the control of which is dependent on NK cell-derived IFN-γ (Dunn and North, 1991). Phenotypically, GATA-3-deficient NK cells lacked expression of Ly49D and appeared less mature, with a reduced percentage of *Gata3*$^{-/-}$ NK cells expressing CD11b and CD43 (Samson et al., 2003). Loss of the zinc-finger transcriptional repressor Blimp-1 also resulted in impaired terminal differentiation of NK cells, as measured by surface expression of CD27, CD11b, and KLRG-1 (Kallies et al., 2011). Though per-cell levels of the cytolytic effector protein granzyme B (Gzmb) were reduced in the absence of Blimp-1, NK-mediated lysis of target cells was unaffected.

Interferon regulatory factors (IRF) share some DNA binding sites with Blimp-1 (Kuo and Calame, 2004), but deletion of IRFs have been reported to affect the development and function of NK cells in distinct ways from deletion of Blimp-1. IRF-1 was reported initially to be required for development and function of NK cells (Duncan et al., 1996; Ohteki et al., 1998), though it was found subsequently that IRF-1 is required for NK cell-extrinsic induction of IL-15 (Ogasawara et al., 1998). The IRF-1-deficient environment was thus inadequate to support development of wild-type-derived NK cells. Conversely, IRF-1-deficient NK cells developed and functioned normally in an IL-15-sufficient environment (Ogasawara et al., 1998). Loss of IRF-2, in contrast, affected the absolute

number and surface phenotype of NK cells in an NK cell-intrinsic fashion (Lohoff et al., 2000).

T-box Transcription Factors in Differentiation of Cytotoxic Lymphocytes

The T-box transcription factor T-bet was found originally to be required for commitment of $CD4^+$ helper T (T_H) cells to the Type 1 (T_H1) lineage, characterized by production of IFN-γ (Szabo et al., 2002). $CD8^+$ cytotoxic T cells also were known to produce IFN-γ, but T-bet was dispensable for this aspect of $CD8^+$ T cell effector function. Eomesodermin (Eomes) is a T-box transcription factor that shares about 75% sequence homology with T-bet in the DNA-binding (T-box) region (Pearce et al., 2003). Consequently, Eomes was found to be responsible for the T-bet-independent production of IFN-γ in $CD8^+$ T cells. It was later observed that T-bet and Eomes redundantly regulate the differentiation of naïve $CD8^+$ T cells to Type 1 effector $CD8^+$ T cells that elaborate IFN-γ and lyse target cells with perforin- and granzyme-containing granules (Intlekofer et al., 2008). In the absence of both T-bet and Eomes, effector $CD8^+$ T cells produce interleukin-17 instead of IFN-γ and exhibit markedly diminished expression of cytolytic proteins.

In addition to directly binding the gene loci for perforin and Gzmb, Eomes binds the promoter region of *Il2rb* and can drive expression of CD122 (Intlekofer et al., 2005). T-bet also drives expression of CD122, but to a lesser extent than does

Eomes. IL-15-responsiveness is a key feature of $CD8^+$ memory T cells (Kennedy et al., 2000), prompting further investigation into the expression patterns of T-bet and Eomes in the effector and memory phases of a $CD8^+$ T cell-mediated immune response. These and subsequent studies revealed non-redundant roles for T-bet and Eomes in specifying the fate of $CD8^+$ effector and memory T cells, respectively. The ratio of T-bet to Eomes expression is highest in $CD8^+$ effector T cells (Intlekofer et al., 2005), consistent with the established role for T-bet in driving activated $CD8^+$ T cells to adopt the effector fate (Intlekofer et al., 2007; Joshi et al., 2007). In contrast, Eomes expression appears to dominate in $CD8^+$ memory T cells (Intlekofer et al., 2005), consistent with the essential role for Eomes in developing or maintaining $CD8^+$ memory T cells that are fit to compete for memory T cell niches (Banerjee et al., 2010). Also independently of T-bet, Eomes is required for the uncontrolled proliferation of aberrant $CD4^-CD8^-$ T cells in the setting of Fas/FasL deficiency (Kinjyo et al., 2010), as well as for the development of innate-like T cells (Gordon et al., 2011).

Functionally, the $CD8^+$ T cell and the NK cell are similar in several ways. Both are cytolytic lymphocytes that can produce copious amounts of IFN-γ. Also, both lineages depend on IL-15 for homeostatic turnover. In light of these similarities, it is not surprising that both killer T cells and NK cells express T-bet and Eomes. Some aspects of NK cell differentiation, function, and homing are known to depend on T-bet. As it has been suggested that T-bet directs CD8+ T cells toward terminal differentiation, so too is T-bet required for the terminal maturation

of NK cells, as measured by upreguation of CD43 and KLRG-1 and downregulation of CD27 (Jenne et al., 2009; Kallies et al., 2011; Soderquest et al., 2011; Townsend et al., 2004). T-bet-deficient NK cells were found to exhibit a modest defect in IFN-γ production and target cell lysis, consistent with the direct regulation of the genes encoding IFN-γ, perforin, and Gzmb by T-bet (Szabo et al., 2002; Townsend et al., 2004). Additionally, a role for T-bet in trafficking of NK cells was recently elucidated. T-bet was found to bind the *S1pr5* gene, encoding the sphingosine-1-phosphate receptor S1P5, which promotes egress from the lymph nodes and bone marrow (Jenne et al., 2009). Roles for Eomes in the differentiation and function of NK cells, however, have not been investigated.

In this thesis work, I investigated the requirements for T-bet and Eomes in the development and function of NK cells. I found novel roles for T-bet and Eomes as complementary gatekeepers of apparently successive checkpoints in the stepwise differentiation of NK cells. Additionally, I have synthesized current prevailing models of NK cell development into a unifying, transcription-based model.

CHAPTER 2

T-BET AND EOMESODERMIN CONTROL KEY CHECKPOINTS OF NATURAL KILLER CELL MATURATION

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INTRODUCTION

NK cells derive from hematopoietic progenitors. A precursor that lacks expression of NK antigens but expresses CD122 (IL-2Rβ/IL-15Rβ) is believed to be committed to the NK lineage and restricted from other blood cell fates (Rosmaraki et al., 2001). NK precursors generate NK antigen-bearing ($NK1.1^+NKp46^+$) immature NK cells that express the integrin α_v and the death ligand TRAIL (Kim et al., 2002; Takeda et al., 2005; Walzer et al., 2007). Immature NK cells give rise to mature NK antigen-bearing cells, which repress integrin a_v and TRAIL and acquire the integrins CD49b ($DX5^+$) and CD11b (Mac-1) (Chiossone et al., 2009; Kim et al., 2002; Takeda et al., 2005). Mature, $DX5^+$ NK cells also express a diverse repertoire of Ly49 family receptors, which are responsible for educating NK cells to self-MHC and for enabling a broad specificity against microbial components (Kim et al., 2002; 2005; Orr and Lanier, 2010).

Development of adult NK cells is thought to take place predominantly in the bone marrow (Di Santo, 2006). NK progenitors and NK developmental intermediates have also been identified in a variety of fetal and adult tissues. Extramedullary niches that appear to foster NK development in mice and in humans include liver, lymph node, and thymus (Freud and Caligiuri, 2006; Luther et al., 2011; Vosshenrich et al., 2006).

The transcription factors Id2, Tox, and E4bp4 (Nfil3) are thought to specify the earliest stages of NK cell development (Aliahmad et al., 2010; Boos et al., 2007; Gascoyne et al., 2009; Kamizono et al., 2009). Loss of various other transcription factors, including Ets-1, Gata-3, and PU.1 has been reported to alter NK cell number and phenotype (Barton et al., 1998; Colucci et al., 2001; Samson et al., 2003). The precise hierarchy of transcription factors governing NK cell maturation, however, is incompletely understood. The highly homologous T-box transcription factors Eomesodermin (Eomes) and T-bet direct fate and function in cytotoxic lymphocytes. Eomes and T-bet redundantly regulate differentiation of $CD8^+$ effector T cells (Intlekofer et al., 2005; 2008; Pearce et al., 2003). Eomes and T-bet also appear to have non redundant functions in specifying the fate of $CD8^+$ T cells (Banerjee et al., 2010; Intlekofer et al., 2007).

It has been suggested that some aspects of terminal NK cell maturation are dependent on T-bet (Jenne et al., 2009; Townsend et al., 2004). We now report an additional role for T-bet in stabilizing immature NK cell development. The consequence of complete deletion of Eomes on NK cell development had not yet been determined. We now show that maturation of NK cells to the stage characterized by expression of the integrin CD49b ($DX5^+$) and acquisition of a diverse repertoire of activating and inhibitory Ly49 family receptors is dependent on Eomes. Maintenance of NK cell maturity was also dependent on Eomes, although maintenance of Ly49 receptor expression occurred independently of Eomes. Progenitors lacking both Eomes and T-bet could not support any NK

lineage development. Taken together, our data support a model in which expression and function of T-bet and Eomes define the key, genetically separable molecular checkpoints of NK cell maturation.

RESULTS

Eomes is required for NK cell maturation

We first examined the expression of Eomes protein at the single-cell level. The majority of NK cells expressed Eomes, but we detected a minor population of NK cells that were Eomes-negative ($Eomes^-$) in each organ examined (**Figure 2.1A**; **Supplementary Figure 2.1A**). The $Eomes^-$ population was most enriched in the liver. A subset of less mature NK cells that express TRAIL and lack CD49b are present in neonates and preferentially reside in the adult liver (Takeda et al., 2005). We found that $Eomes^-$ cells are characterized by TRAIL expression, whereas $Eomes^+$ cells are typified by CD49b expression ($DX5^+$) (**Figure 2.1A**). $Eomes^-$ and $Eomes^+$ NK cells also expressed different repertoires of homing receptors, consistent with their disparate anatomic localization. $Eomes^-$ but not $Eomes^+$ NK cells expressed the Integrin α_v and the chemokine receptors, CXCR3 and CXCR6 (**Figures 2.1B** and **2.1C**).

To understand the requirements for Eomes in the development and homeostasis of NK cells, we utilized a hematopoietic-specific knockout of *Eomes*. These mice harbored floxed alleles of Eomes (Intlekofer et al., 2008) and expressed Cre recombinase under control of *Vav* regulatory elements (Stadtfeld, 2004). *Eomes*$^{Fl/Fl}$*, Vav-Cre*$^+$ (Eomes cKO) mice had a substantial reduction of NK cells in the spleen and the blood, relative to wild-type animals (**Figure 2.1D**; **Supplementary Figure 2.1B**). We observed a more modest reduction in NK cell number in the liver, lymph node, and bone marrow of Eomes cKO mice. Deletion

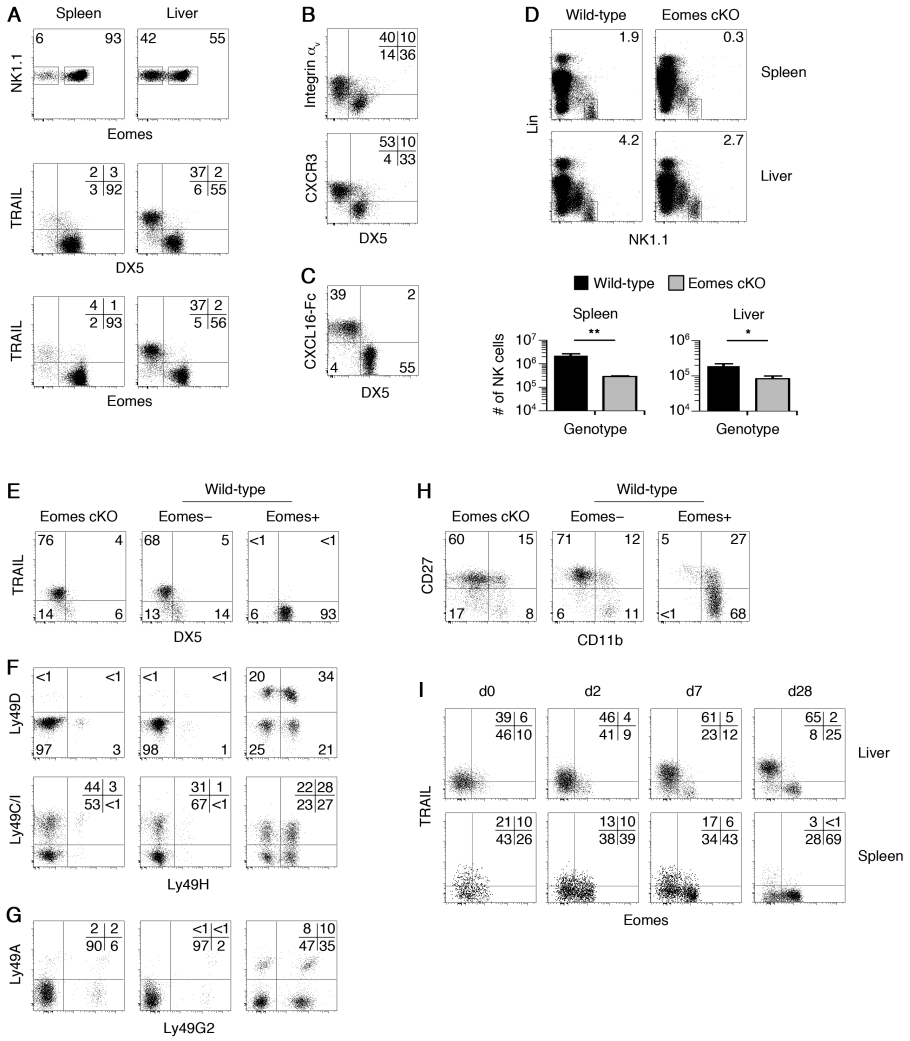
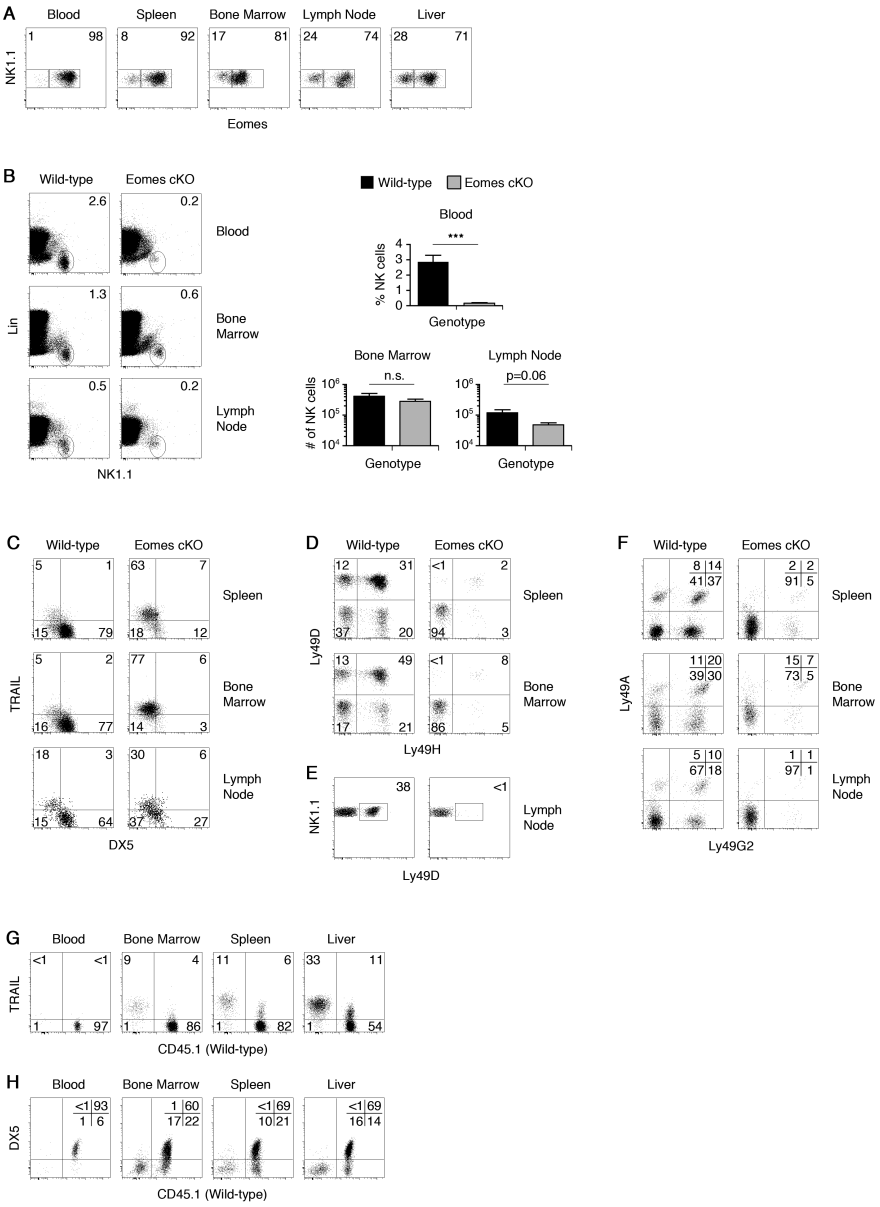


Figure 2.1. Eomes required for maturation of NK cells
(**A**) Expression of Eomes and maturation markers TRAIL and DX5 in wild-type splenic and hepatic NK cells. Plots represent $CD3e^{-}CD19^{-}NK1.1^{+}NKp46^{+}CD122^{+}$ cells. Data are representative of at least 5 separate experiments. (**B**) Integrin a_v and CXCR3 expression on wild-type hepatic NK cells. Data are representative of at least 5 separate experiments. (**C**) CXCR6 expression on wild-type hepatic NK cells. CXCL16-Fc binds to surface CXCR6 (Matloubian et al., 2000). CXCL16-Fc was detected by secondarily adding a biotin-conjugated, anti-human Fc antibody, followed by staining with fluorochrome-conjugated streptavidin. Data are representative of 3 separate experiments. (**D**) Analysis of splenic and hepatic NK compartments in wild-type and Eomes cKO mice, with quantification of absolute numbers of NK cells. Data are representative of at least 5 independent experiments. Error bars indicate standard deviation. *, $p<0.05$; **, $p<0.001$. (**E**) Maturation markers TRAIL and DX5 in Eomes cKO, wild-type $Eomes^{-}$, and wild-type $Eomes^{+}$ NK cells. Data are representative of 5 separate experiments. (**F** and **G**) Ly49 repertoire of Eomes cKO NK cells, compared to wild-type $Eomes^{-}$ and $Eomes^{+}$ NK cells. Data are representative of at least 3 separate experiments. (**H**) Maturation markers CD27 and CD11b in Eomes cKO, wild-type $Eomes^{-}$, and wild-type $Eomes^{+}$ NK cells. Data are representative of at least 3 separate experiments. (**I**) Kinetic analysis of Eomes expression in NK cells of mice of indicated ages. Day 0 (d0) denotes the day of birth. Data are representative of 2 separate experiments.



Supplementary Figure 2.1. Cell-intrinsic requirement for Eomes in maturation of NK cells (**A**) Expression of Eomes in wild-type NK cells of indicated organs. Data are representative of at least 5 separate experiments. (**B**) Analysis and quantification of the NK compartments in indicated organs of wild-type and Eomes cKO mice. Data are representative of 3-5 independent experiments. Error bars indicate standard deviation. n.s., not significant, $p>0.05$; ***, $p<0.001$. (**C**) Maturation marker expression on wild-type and Eomes cKO NK cells in indicated organs. (**D**, **E**, and **F**) Ly49 repertoire expression on wild-type and Eomes cKO NK cells. Data are representative of at least 3 separate experiments. (**G** and **H**) Analysis of maturation markers TRAIL (**G**) and DX5 (**H**) on NK cells isolated from wild-type (CD45.1+) plus Eomes cKO (CD45.2+) mixed bone marrow chimeras, 6-10 weeks post-bone-marrow-transplant. Data are representative of analyses from at least 3 individual mice.

of Eomes appeared to affect NK numbers in organs that normally contain greater numbers of mature, $DX5^+$ NK cells. We, therefore, interrogated the phenotype of NK cells remaining in the absence of Eomes. NK cells from Eomes cKO mice approximated the phenotype of immature, $Eomes^-$ NK cells from wild-type mice, expressing TRAIL but lacking CD49b as well as Ly49A, Ly49D, Ly49G2, and Ly49H (**Figures 2.1E**, **2.1F**, and **2.1G**; **Supplementary Figures 2.1C**, **2.1D**, **2.1E**, and **2.1F**). NK cells from Eomes cKO mice and immature, $Eomes^-$ NK cells from wild-type mice do, however, efficiently express Ly49C and/or Ly49I (**Figure 2.1F**). Eomes cKO and wild-type $Eomes^-$ NK cells also shared the immature, $CD27^{high}CD11b^{lo}$ phenotype (**Figure 2.1H**). The requirement for Eomes to develop mature, $DX5^+$ NK cells appeared to be cell-intrinsic. Wild-type plus Eomes cKO mixed bone marrow chimeras were generated and analyzed for NK cell composition. Eomes cKO marrow efficiently contributed to the immature, $TRAIL^+$ NK cell lineage, but Eomes-deficient marrow did not contribute to the mature, $DX5^+$ NK cell compartment (**Supplementary Figures 2.1G** and **2.1H**). These data suggest that Eomes is an intrinsic and non-redundant regulator of the genetic program of NK cell maturation.

Neonatal NK cells lack markers of maturity and express limited Ly49 receptor repertoires (Kubota et al., 1999; Takeda et al., 2005), somewhat akin to NK cells in adult Eomes cKO mice. We observed that NK cells from neonatal mice have minimal expression of Eomes (**Figure 2.1I**). We also found that postnatal NK cells in the spleen initiated Eomes expression more readily than did hepatic

neonatal NK cells. The delayed onset and anatomic bias of Eomes expression is consistent with prior developmental kinetic analysis indicating that NK maturation to the $DX5^+$ stage is evident after the postnatal period and occurs preferentially outside the liver (Takeda et al., 2005). Whether the early splenic environment contains signals that promote the induction of Eomes or simply lacks hepatic signals that restrict Eomes induction remains to be determined. Our data support a model wherein progenitors lacking Eomes arrest at an immature stage of NK cell development, unable to progress to the Ly49- and CD49b-expressing ($DX5^+$) stage of maturation that characterizes most adult NK cells.

$Eomes^-$ NK cells can give rise to $Eomes^+$ NK cells

Eomes appears to direct NK maturation past the $TRAIL^+$Integrin α_v^+ state to the CD49b-expressing ($DX5^+$) state. Whether $Eomes^-$ NK cells are the direct predecessors of $Eomes^+$ NK cells has not been determined. Analyses of NK cell developmental stages in the bone marrow and cell transfer experiments using defined intermediates are compatible with a model wherein $TRAIL^+$Integrin α_v^+ NK cells may give rise to $DX5^+$ NK cells (Chiossone et al., 2009; Kim et al., 2002; Takeda et al., 2005). To address whether $TRAIL^+$ NK cells can upregulate Eomes while maturing into $DX5^+$ NK cells, we FACS-sorted immature, $TRAIL^+$ NK cells to 97% purity and transferred them into unirradiated, immunodeficient *Il2rg*$^{-/-}$, *Rag2*$^{-/-}$ (Gamma-common, Rag double KO) mice, which lack NK cells and lymphocytes. One (**Figure 2.2A**) or two (**Figure 2.2B**) weeks following adoptive transfer, the livers, spleens, and bone marrow of Gamma-common, Rag double

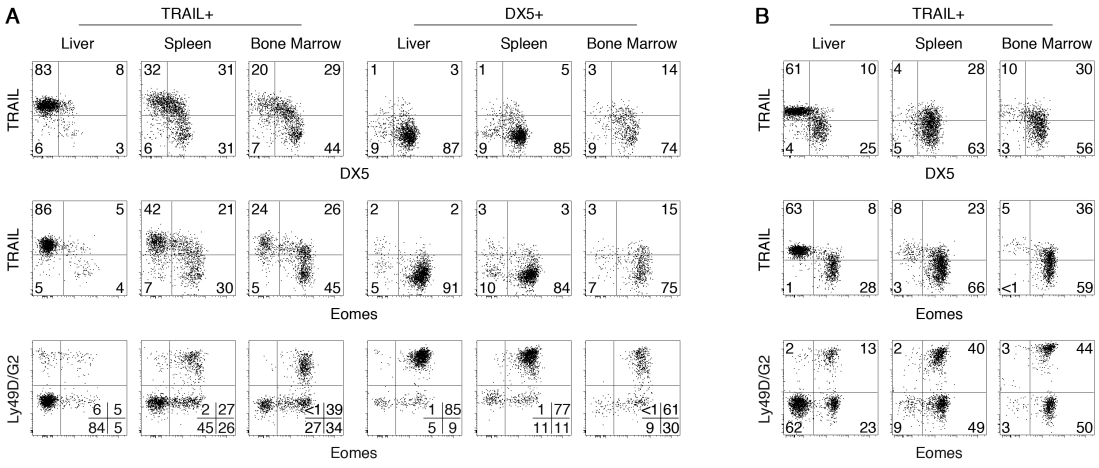


Figure 2.2. Eomes^{-} NK cells can give rise to Eomes^{+} NK cells
Highly purified TRAIL^{+} and DX5^{+} donor NK cells were adoptively transferred into Gamma-common, Rag double KO recipients. One (**A**) or two (**B**) weeks post-transfer, indicated organs of recipient mice were harvested and NK cells were analyzed for expression of Eomes, Ly49 receptors, and maturation markers TRAIL and DX5. Data are representative of 3 separate experiments.

KO recipients were analyzed for the presence of NK cells. We found that DX5$^+$ NK cells remain DX5$^+$, Eomes$^+$, and Ly49$^+$ after adoptive transfer (**Figures 2.2A** and **2.2B**), consistent with prior experiments showing stability of CD49b expression (Takeda et al., 2005). Acquisition of Eomes expression in the mature, DX5$^+$ stage of NK cell development, thus, appears to be a stable event.

Transferred immature, TRAIL$^+$ NK cells underwent *in vivo* maturation characterized by repression of TRAIL and induction of CD49b (DX5$^+$), Eomes, and Ly49 receptors (**Figures 2.2A** and **2.2B**). NK cell maturation and induction of Eomes was restricted in the liver, promoted in the spleen, and favored most in the bone marrow. The anatomical hierarchy of induction of Eomes in the NK cell lineage mirrors the findings that NK cell maturation is a developmentally regulated event. Expression of Eomes and maturation from TRAIL$^+$ to the DX5$^+$ Ly49$^+$ NK cell lineage appears to be delayed until the postnatal period, when the primary site of hematopoiesis shifts from the liver to the bone marrow (Keller et al., 1999).

Eomes maintains maturity of NK cells

Induction of Eomes appears to be required to establish the mature NK cell state. To investigate the role of Eomes in maintaining NK cells in the mature state, we temporally deleted Eomes from mature NK cells. Splenic NK cells with floxed alleles of *Eomes* were isolated and treated directly *ex vivo* with Cre-recombinase fused to the transduction domain of the HIV TAT protein (TAT-Cre) (Wadia et al.,

2004). Cells were exposed to TAT-Cre for 45 minutes to allow TAT to chaperone Cre across the cell and nuclear membranes. Intranuclear Cre protein could then excise the floxed portion of *Eomes*. The protein transduction reaction was quenched by addition of fetal bovine serum followed by thorough washing. Treated or sham-treated cells were then adoptively transferred into unirradiated Gamma-common, Rag double KO mice.

One week after cell transfer, we analyzed the NK cell compartments of recipient mice. From recipients of sham-treated cells, we retrieved mature, $Eomes^{+}DX5^{+}$ NK cells that expressed diverse Ly49 receptors and immature, $Eomes^{-}TRAIL^{+}$ NK cells that displayed a paucity of Ly49 receptor expression (**Figures 2.3A** and **2.3B**). Based on the failure of $Eomes^{+}DX5^{+}$ NK cells to convert to $Eomes^{-}$ $TRAIL^{+}$ NK cells (**Figure 2.2**), we attribute the presence of a sizeable $Eomes^{-}$ population to vigorous expansion of pre-existing $Eomes^{-}TRAIL^{+}$ NK cells in the donor organs. From recipients of TAT-Cre-treated cells, we retrieved an additional population of $Eomes^{-}$ cells that expressed Ly49 receptors. Because the $Eomes^{-}Ly49^{+}$ state did not arise in sham-treated cells, this subset presumably represented the formerly mature, $Eomes^{+}DX5^{+}$ NK cells that underwent Cre-mediated deletion of Eomes (**Figures 2.3A** and **2.3B**). The stability of all Ly49 family members examined in cells lacking Eomes protein over the interval of the cell transfer suggested that maintenance of Ly49 receptor expression may not be dependent on Eomes, even though establishment of Ly49 receptor expression appeared to be dependent on Eomes.

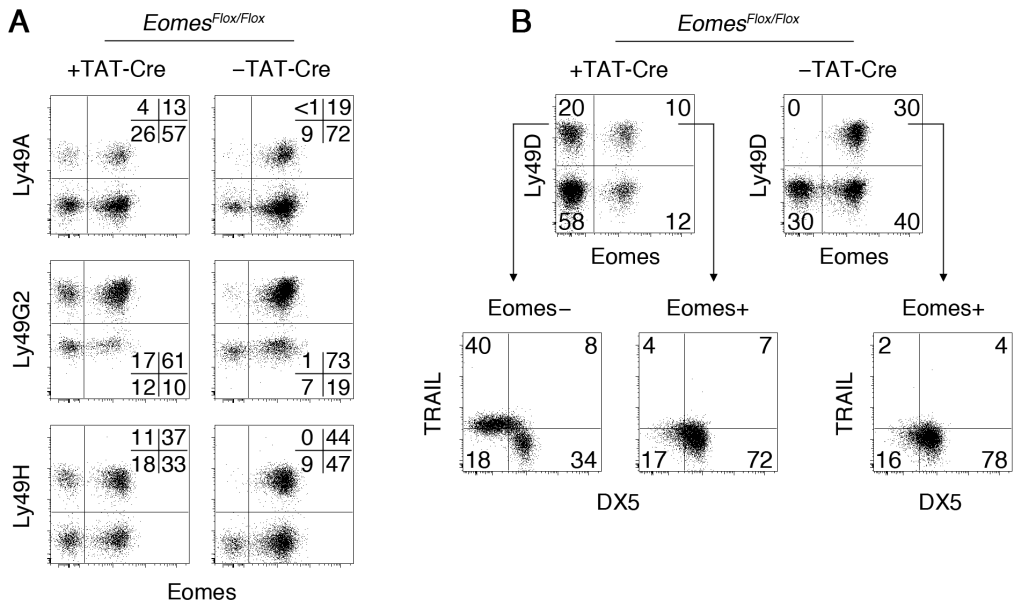
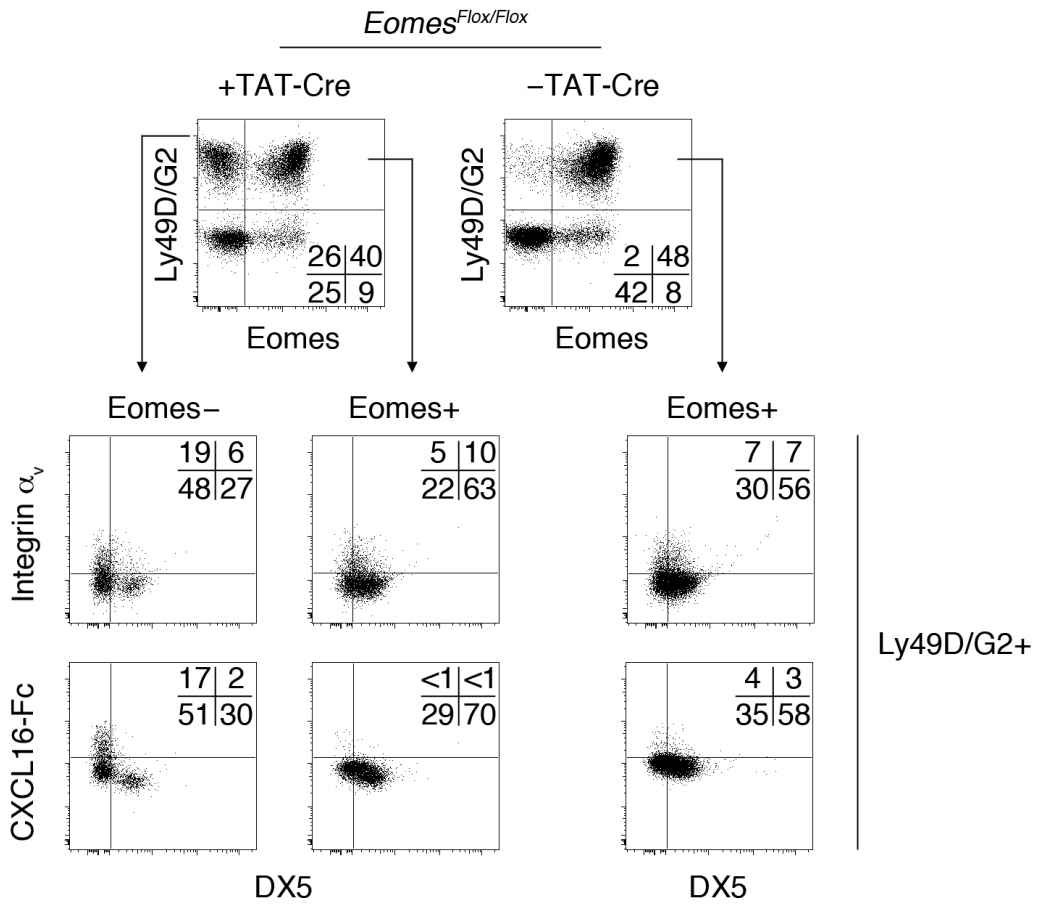


Figure 2.3. Eomes required to maintain some aspects of mature NK cells
Splenic NK cells with floxed alleles of *Eomes* were purified and either treated with TAT-Cre or sham-treated directly *ex vivo*. Donor cells were transferred into Gamma-common, Rag double KO recipients and analyzed 7-10 days later. (**A**) NK cells from spleens of recipient mice were analyzed for expression of Eomes and indicated Ly49 receptors. Data are representative of at least 2 separate experiments. (**B**) NK cells from livers of recipient mice were analyzed for Ly49 receptor expression (top row). Ly49+ NK cells were also analyzed for maturation markers TRAIL and DX5 (bottom row). Data are representative of 5 separate experiments.

In contrast to the stability of Ly49 expression, we found that temporal deletion of Eomes resulted in loss of many of the other markers of maturity. Ly49^{+} NK cells that retained expression of Eomes after treatment with TAT-Cre exhibited a mature phenotype (TRAIL^{-}DX5^{+}) (**Figure 2.3B**). A substantial proportion of Ly49^{+} NK cells that lost expression of Eomes became TRAIL^{+} and DX5^{-}. We also observed partial derepression of Integrin α_v and CXCR6 in Ly49^{+} NK cells after loss of Eomes (**Supplementary Figure 2.2**). These findings suggest that Eomes directly or indirectly represses the immaturity markers TRAIL, Integrin α_v, and CXCR6, while inducing the maturity marker CD49b (DX5^{+}). Mature, Eomes^{+}DX5^{+} NK cells normally retain their TRAIL^{-}DX5^{+} identity (**Figure 2.2**), but loss of Eomes can cause mature cells to become immature, TRAIL^{+} DX5^{-}, despite their maintenance of Ly49 receptors (**Figure 2.3B**). The reversion in attributes after temporal deletion of Eomes complements the prior findings suggesting that immature, Eomes^{-}TRAIL^{+} NK cells can be *in vivo* intermediates that give rise to mature Eomes^{+}DX5^{+} NK cells (**Figure 2.2**). Together, these experiments provide forward and reverse evidence that acquisition of Eomes elaborates and maintains mature attributes onto the immature NK cell foundation.

T-bet stabilizes Eomes^{-} NK cells

To evaluate the role of T-bet in NK cell development, we compared the phenotypes and patterns of T-box protein expression in NK lineages of Eomes cKO and *Tbx21*$^{-/-}$ (T-bet KO) mice. Tissues from T-bet KO mice only contained NK cells expressing CD49b (DX5^{+}), a diverse Ly49 repertoire, and high levels of



Supplementary Figure 2.2. Eomes maintains repression of immature NK cell markers
Splenic NK cells with floxed alleles of *Eomes* were purified and either treated with TAT-Cre or sham-treated directly *ex vivo*. Donor cells were transferred into Gamma-common, Rag double KO recipients and analyzed 7-10 days later. NK cells from livers of recipient mice were analyzed for expression of Eomes and Ly49D and/or Ly49G2 (top row). Ly49D+ and/or Ly49G2+ NK cells were also analyzed for expression of integrin αv (middle row) and CXCR6 (bottom row). Data are representative of 2 separate experiments.

Eomes (**Figures 2.4A** and **2.4B**; **Supplementary Figures 2.3A** and **2.3B**). Immature, Eomes^{-}TRAIL^{+} NK populations were seemingly absent from T-bet KO mice, while TRAIL^{+} cells represented the predominant NK population of Eomes cKO mice. T-bet KO and Eomes cKO mice thus have reciprocal maturational phenotypes: T-bet KO mice lack immature (DX5^{-}) cells, while Eomes cKO mice lack mature (DX5^{+}) NK cells.

T-bet is normally expressed in both Eomes^{-} and Eomes^{+} NK cells (**Figure 2.4B**; **Supplementary Figure 2.3A**), which is consistent with roles for T-bet in both immature and mature NK cell stages. In addition to playing an essential role in stabilizing the immature, DX5^{-} subset (**Figure 2.4A)**, T-bet controls repression of CD27 and induction of CD43 in mature, CD11b^{high}DX5^{+} NK cells (**Supplmentary Figure 2.3C**), as has been previously suggested (Jenne et al., 2009; Townsend et al., 2004). Although mature, DX5^{+} NK cells can develop without T-bet, the terminal stages of maturation are incomplete in the absence of T-bet. Using wild-type plus T-bet KO mixed bone marrow chimeras, we found that the previously described role for T-bet in repressing CD27 among mature NK cells and the newly described role for T-bet in development or maintenance of the immature, TRAIL^{+}Eomes^{-}DX5^{-} NK population were both cell-intrinsic effects (**Supplementary Figures 2.3D**, **2.3E,** and **2.3F**).

The lack of immature, TRAIL^{+} NK cells in the absence of T-bet could be consistent with a role for T-bet in the development or maintenance of TRAIL^{+} NK

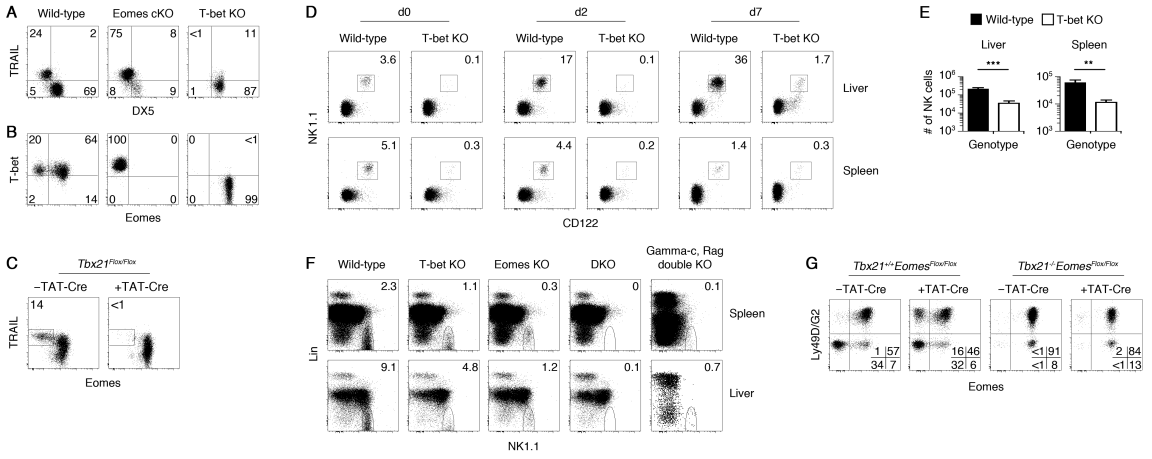
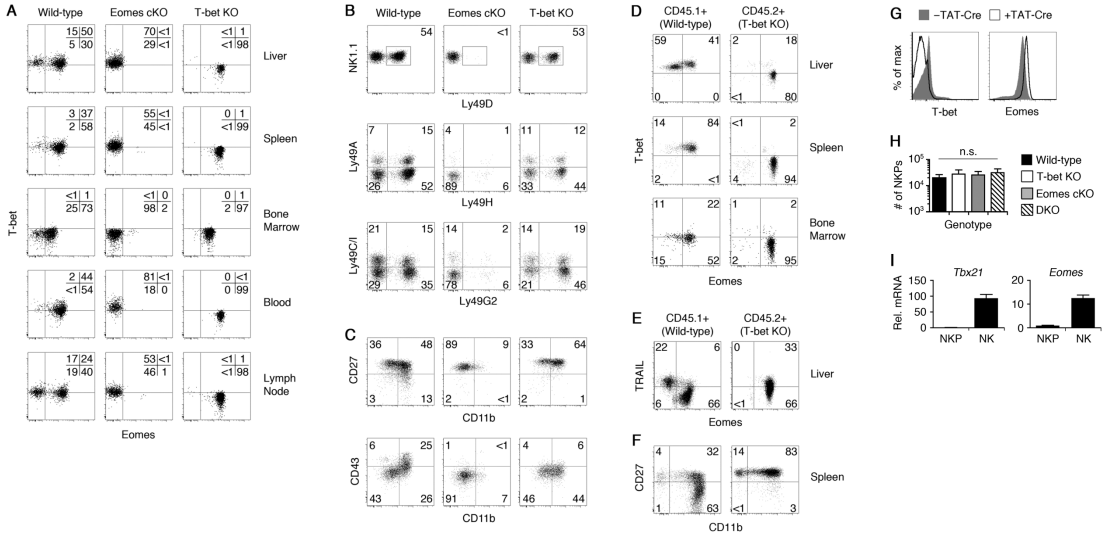


Figure 2.4. T-bet stabilizes immature, Eomes^{-} NK cells
(**A** and **B**) Expression of maturation markers TRAIL and DX5, along with expression of T-bet and Eomes, in wild-type, Eomes cKO, and T-bet KO hepatic NK cells. Data are representative of at least 5 separate experiments. (**C**) Effect of acute loss of T-bet in NK cells. Hepatic NK cells with floxed alleles of *Tbx21* were purified and either treated with TAT-Cre or sham-treated directly *ex vivo*. Donor cells were transferred into Gamma-common, Rag double KO recipients and analyzed 7-10 days later. NK cells from livers of recipient mice were analyzed for expression of Eomes and Ly49 receptors. Data are representative of 2 separate experiments. (**D**) Developmental analysis of hepatic and splenic NK cell compartments in wild-type and T-bet KO mice of indicated ages. Day 0 (d0) denotes the day of birth. Data are representative of at least 5 separate experiments. (**E**) Quantification of absolute number of NK cells in wild-type and T-bet KO neonatal mice, aged d0 to d2. Error bars indicate standard deviation. **, p<0.001; ***, p<0.0001. (**F**) Effect of loss of T-bet and/or Eomes on hepatic and splenic NK cell compartments. Data are representative of 2 separate experiments. (**G**) Effect of acute loss of Eomes in T-bet-deficient NK cells. Splenic *Tbx21*$^{+/+}$ or *Tbx21*$^{-/-}$ NK cells with floxed alleles of *Eomes* were purified and either treated with TAT-Cre or sham-treated directly *ex vivo*. Donor cells were transferred into Gamma-common, Rag double KO recipients and analyzed 7-10 days later. NK cells from livers of recipient mice were analyzed for expression of Eomes and Ly49 receptors. Data are representative of 2 separate experiments.



Supplementary Figure 2.3. T-bet stabilizes Eomes$^-$ NK cells in a cell-intrinsic fashion but does not specify NK precursors

(**A**) T-bet and Eomes expression in NK cells isolated from indicated organs of wild-type, Eomes cKO, and T-bet KO mice. Data are representative of at least 3 separate experiments. (**B**) Ly49 repertoire of splenic NK cells from mice of indicated genotypes. Data are representative of at least 3 separate experiments. (**C**) Expression of maturation markers CD11b, CD27, and CD43 on bone marrow-resident NK cells from mice of indicated genotypes. Data are representative of at least 3 separate experiments. (**D**, **E**, and **F**) Expression of T-bet and Eomes (**D**), as well as maturation markers TRAIL (**E**), CD27 and CD11b (**F**), in NK cells isolated from indicated organs of wild-type (CD45.1$^+$) plus Eomes cKO (CD45.2$^+$) mixed bone marrow chimeras, 6-10 weeks post-bone-marrow-transplant. Data are representative of analyses from 3-5 individual mice. (**G**) Effect of acute loss of T-bet on Eomes expression. Hepatic NK cells with floxed alleles of *Tbx21* were purified and either treated with TAT-Cre or sham-treated directly *ex vivo*. Donor cells were transferred into Gamma-common, Rag double KO recipients and analyzed 7-10 days later. Hepatic NK cells were analyzed for expression of Eomes. Data are representative of 2 separate experiments. (**H**) Quantification of NK precursors (NKPs, intracellular and extracellular CD3ε$^-$, CD19$^-$Gr-1$^-$Ter119$^-$NK1.1$^-$DX5$^-$, CD122^{high}) cells in wild-type, T-bet KO, Eomes cKO, and DKO mice. Error bars indicate standard deviation. n.s., not significant, p>0.05. (**I**) NK1.1$^-$ NK precursor cells (NKP) and NK1.1$^+$ NK cells (NK) were purified from wild-type bone marrow and analyzed for expression of *Tbx21* and *Eomes* mRNA by qRT-PCR.

cells. We tested whether T-bet is required for the stability of the TRAIL$^+$ subset. T-bet was temporally deleted by treating hepatic NK cells harboring floxed alleles of *Tbx21* with TAT-Cre *ex vivo*. Treated or sham-treated cells were adoptively transferred into unirradiated Gamma-common, Rag double KO mice. One week later, we harvested and analyzed the NK compartments of the recipients. From recipients of sham-treated cells, we recovered both immature, Eomes$^-$TRAIL$^+$ NK cells and mature, Eomes$^+$ NK cells (**Figure 2.4C**). From recipients of TAT-Cre-treated cells, we detected a sizeable population of NK cells that lost expression of T-bet (**Supplementary Figure 2.3G**). TAT-Cre-treatment markedly diminished the presence of immature, Eomes$^-$TRAIL$^+$ NK cells while not seemingly affecting the recovery of mature, Eomes$^+$ NK cells (**Supplementary Figure 2.4C**). Whether the loss of T-bet simply caused immature, Eomes$^-$ TRAIL$^+$ cells to perish or, instead, catalyzed their conversion to mature, Eomes$^+$ NK cells has not been determined. Accelerated maturation from the loss of T-bet might be possible owing to the finding that NK cells from which T-bet was acutely (**Supplementary Figure 2.3G**) or chronically (**Supplementary Figure 2.3A**) deleted both exhibited increased levels of Eomes expression. Whether by enabling its formation, maintaining its survival, or curbing its progressive maturation, the present findings suggest that T-bet stabilizes the immature, Eomes$^-$TRAIL$^+$ stage of NK cell development.

T-bet appears required to stabilize immature NK cells, and immature NK cells are the predominant NK cells in the perinatal period (Takeda et al., 2005). We,

therefore, examined the NK compartment in neonatal mice. T-bet KO neonates possessed substantially reduced NK cell numbers relative to age-matched, wild-type control mice (**Figures 2.4D** and **2.4E**). These data support a model in which immature neonatal NK cells depend on T-bet for development or stability. Because the neonatal environment is restrictive for Eomes induction, there does not appear to be precociously accelerated NK cell maturity at this stage of life.

T-bet or Eomes is required for an NK lineage cell to be maintained

The data presented thus far suggest that T-bet and Eomes are required to stabilize sequential or alternative stages of NK cell maturation. We intercrossed Eomes cKOs with T-bet KOs to yield *Tbx21*$^{-/-}$*Eomes*$^{Flox/Flox}$, *Vav-Cre*$^{+}$ (DKO) animals, lacking both Eomes and T-bet in the hematopoietic compartment. We could not detect NK cells in any organ of the DKO animals (**Figure 2.4F**). Despite the absence of NK antigen-expressing NK lineage cells, we found that NK antigen-negative precursors of NK cells were present in DKO mice (**Supplementary Figure 2.3H**). The apparent lack of requirement of T-bet and Eomes to develop NK lineage-committed precursors is consistent with the low levels of T-bet and Eomes gene expression in wild-type NK precursors (**Supplementary Figure 2.3I**).

Eomes-dependent NK cells can revert to an apparently less mature state, after loss of Eomes (**Figure 2.3**). To determine whether this reverted state is T-bet-dependent, we temporally deleted floxed alleles of *Eomes* on a T-bet KO

background. *Eomes*$^{flox/flox}$*Tbx21*$^{-/-}$ cells were treated with TAT-Cre *ex vivo*, washed, and adoptively transferred into unirradiated Gamma-common, Rag double KO mice. Treated *Eomes*$^{flox/flox}$ NK cells on a T-bet-sufficient background lost expression of Eomes and persisted in recipient mice (**Figure 2.4G**). By contrast, T-bet-deficient NK cells that underwent temporal deletion of Eomes could not exist as a stable population in recipient mice. Thus, the absence of T-bet and Eomes results in the loss of immature and mature NK cells, respectively. The absence of both factors, however, is incompatible with a stable NK-antigen-bearing NK cell.

Expression of effector genes by Eomes^{-} and Eomes^{+} NK cells

Eomes^{-}TRAIL^{+} and Eomes^{+}DX5^{+} NK cells are dependent on distinct T-box transcription factors and exhibit distinct surface phenotypes. Whether immature and mature NK cells express similar functional genes has not been fully determined. NK cells kill targets by releasing cytotoxic granules or engaging death receptors (Smyth et al., 2005). We examined the expression of molecular effectors of cytolysis in immature and mature NK cells. Quantitative, real-time, reverse transcriptase PCR (qRT-PCR) was performed on purified TRAIL^{+} and DX5^{+} NK cells. We found that TRAIL^{+} cells expressed moderately less Perforin (*Prf1*) mRNA than DX5^{+} cells (**Figure 2.5A**), consistent with a role for Eomes in inducing *Prf1* (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005; Pearce et al., 2003). Despite the reduction relative to DX5^{+} NK cells, levels of *Prf1* in TRAIL^{+} NK cells were higher than in naïve CD8^{+} T cells (**Supplementary Figure 2.4A**).

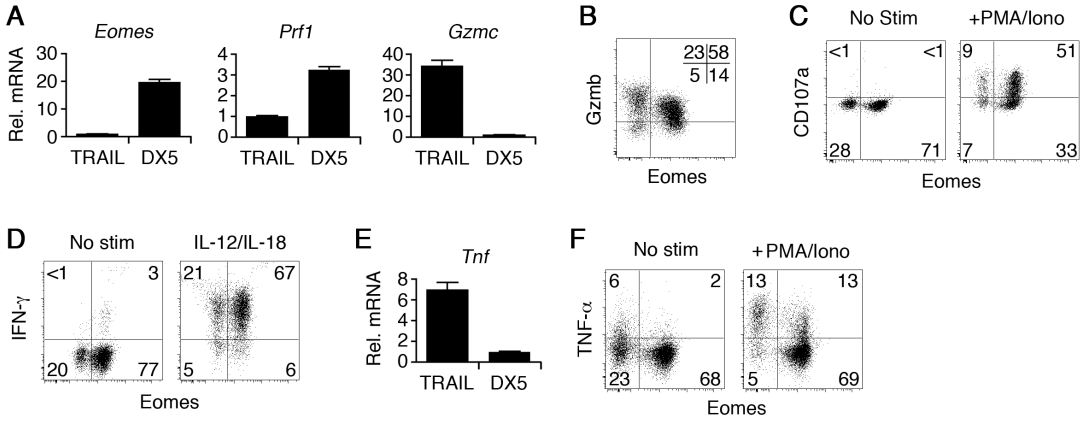
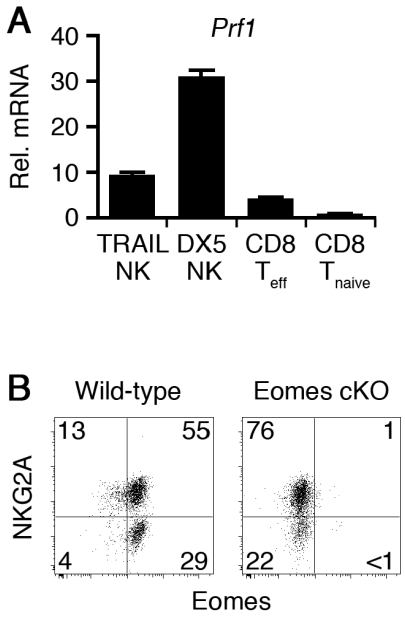


Figure 2.5. Effector gene expression of Eomes$^-$ and Eomes$^+$ NK cells
(**A**) *Eomes*, *Prf1*, and *Gzmc* mRNA expression in purified TRAIL$^+$ and DX5$^+$ NK cells. Error bars indicate standard error of the mean (SEM). Data are representative of 3 separate experiments. (**B**) Expression of Granzyme B (Gzmb) in wild-type, unstimulated hepatic NK cells. Data are representative of 3 separate experiments. (**C**) Analysis of degranulation by wild-type hepatic NK cells *in vitro.* Hepatic lymphocytes were isolated and stimulated with PMA/Iono for 4 hours *in vitro* in the presence of anti-CD107a antibody. Data are representative of 3 separate experiments. (**D**) Expression of IFN-γ in wild-type hepatic NK cells after stimulation with recombinant IL-12 and IL-18 for 4 hours *in vitro*. Data are representative of 3 separate experiments. (**E**) *Tnf* mRNA expression in wild-type, unstimulated, highly-purified TRAIL$^+$ and DX5$^+$ NK cells. Error bars indicate standard error of the mean (SEM). Data are representative of 3 separate experiments. (**F**) Expression of TNF-α in wild-type hepatic NK cells after stimulation with PMA/Iono for 4 hours *in vitro*. Data are representative of 3 separate experiments.



Supplementary Figure 2.4. Expression of effector molecules and inhibitory receptors by Eomes$^-$ and Eomes$^+$ NK cells

(**A**) Expression of *Prf1* mRNA by qRT-PCR in indicated groups of cells. Wild-type TRAIL$^+$ NK cells and DX5$^+$ NK cells were purified from the livers of uninfected mice. CD8$^+$ naïve (CD44^{lo}CD62L^{hi}) T cells were purified from the spleens of uninfected mice. CD8$^+$ effector T (CD44^{hi}gp33-Tetramer$^+$) cells were purified from the spleens of mice infected 8 days prior with lymphocytic choriomeningitis virus. Data are representative of 2 separate experiments. (**B**) NKG2A expression on splenic NK cells from wild-type and Eomes cKO mice. Data are representative of 2 separate experiments.

TRAIL^{+} NK cells expressed increased levels of Granzyme C (*Gzmc*) mRNA, relative to Eomes^{+}DX5^{+} NK cells (**Figure 2.5A**). Both subsets from the liver stained brightly for Granzyme B protein (**Figure 2.5B**). To test whether Eomes^{-} TRAIL^{+} NK cells can degranulate, we stimulated hepatic lymphocytes briefly with phorbol myristolate acetate and ionomycin (PMA/Iono) *in vitro*. Cells were cultured in the presence of anti-CD107a (lysosomal-associated membrane protein-1, LAMP-1) antibody to assess degranulation (Alter et al., 2004). We found that both Eomes^{-} and Eomes^{+} NK cells efficiently de-granulate (**Figure 2.5C**).

NK cells also release proinflammatory cytokines, such as IFN-γ and TNF-α, in response to stimuli (Vivier et al., 2011). We found that Eomes^{-} and Eomes^{+} NK cells express IFN-γ after stimulation with IL-12/IL-18 (**Figure 2.5D**), consistent with a prior analysis of TRAIL^{+} versus DX5^{+} NK cells (Takeda et al., 2005). The Eomes^{-}TRAIL^{+} subset expressed higher levels of basal TNF-α (*Tnf*) mRNA (**Figure 2.5E**) and contained higher levels of TNF-α protein after stimulation with PMA/Iono (**Figure 2.5F**).

NK cells specifically kill target cells and spare healthy host cells (Orr and Lanier, 2010). Various receptors, including Ly49 family members and NKG2A, have been implicated in educating NK cells to self. NKG2A binds the nonclassical MHC class I molecule Qa-1^{b} and may participate in tolerizing NK cells to self (Vance et al., 1998). Neonatal NK cells express inhibitory receptors but lack

activating receptors (Kubota et al., 1999). Eomes$^-$ NK cells expressed similar levels of NKG2A and at similar frequencies to Eomes$^+$ NK cells (**Supplementary Figure 2.4B**). We found that Eomes$^-$ NK cells also expressed Ly49C and/or Ly49I (**Figure 2.1**), which bind MHC Class I ligands in C57BL/6 mice (Brennan et al., 1996a; 1996b; Liu et al., 2000). Immature, Eomes$^-$ NK cells, thus, appear to be educated to self MHC and licensed to identify and kill targets that are missing self (Kim et al., 2005), which is consistent with prior analysis suggesting that both TRAIL$^+$ and DX5$^+$ NK cells can lyse YAC-1 target cells *in vitro* (Takeda et al., 2005) and our own preliminary findings that Eomes-deficient mice can clear target cells that are missing self *in vivo*. The functional significance of the observed differences in expression of effector molecules by immature and mature NK cells (Perforin, Granzyme C, TNF-α, etc.) has yet to be determined.

DISCUSSION

Developing NK cells are believed to derive from lymphoid progenitors (Spits and Di Santo, 2011). Restriction of B cell potential and commitment to the innate lymphoid lineage appears to be mediated by Id2 (Boos et al., 2007; Carotta et al., 2011). Id2-expressing NK precursors may give rise to canonical NK cells in an E4bp4- and Tox-dependent fashion (Aliahmad et al., 2010; Carotta et al., 2011; Gascoyne et al., 2009; Kamizono et al., 2009). Previous studies have elucidated various phenotypic stages of NK cell development and maturation (Chiossone et al., 2009; Kim et al., 2002; Takeda et al., 2005), but the molecular events that control passage through these stages are not fully understood.

We found that NK cell development is profoundly impaired in the absence of both Eomes and T-bet. The phenotypes of T-bet KO mice and Eomes cKO mice are not, however, identical. Each single KO mouse exhibited a substantial loss of NK cells. The most striking defect in each case was the loss of a distinct and non-overlapping phenotypic subset from that lacking in the other knockout mouse. Roughly, the double KO phenotype approximated the addition of the two single KO phenotypes.

Our data support a model wherein NK precursors may upregulate T-bet and transit to an immature, $Eomes^{-}DX5^{-}TRAIL^{+}T\text{-}bet^{+}$ state. This developmental sequence seems to be the predominant, if not exclusive, manner of NK cell development in embryogenesis and in neonates, when hematopoiesis is

predominantly occurring in the liver (Keller et al., 1999). Immature-phenotype, Eomes^{-}DX5^{-}TRAIL^{+}T-bet^{+} NK cells persist in adulthood, predominantly as a stable lineage in the liver and as a more mercurial intermediate that transits to a mature stage in the bone marrow (Kim et al., 2002; Takeda et al., 2005). Importantly, the immature, DX5^{-}TRAIL^{+} NK cell identity appears strictly dependent on T-bet, but not Eomes, for its stability. Whether immature NK cells in the adult liver arise *de novo* from NK precursors in the adult liver, represent homeostatic divisions of immature NK cells that developed early in life, are migrants from the bone marrow, or possess some other ontogenic history remains to be determined. Transfer studies presented herein indicate that the liver remains a non-permissive environment for induction of Eomes in NK cells throughout the lifetime of the organism.

Our findings support a model for a different scenario in adult hematopoiesis, which occurs predominantly in the bone marrow. NK precursors appear to give rise to a similarly immature-appearing, Eomes^{-}DX5^{-}TRAIL^{+}T-bet^{+} stage. This intermediate appears to yield subsequent stages of maturation that we now show are strictly dependent on the expression and function of Eomes. The Eomes-dependent phenotypic properties of mature, Eomes^{+}DX5^{+}TRAIL^{-}T-bet^{+} NK cells, including repression of TRAIL and expression of CD49b (DX5^{+}), also require ongoing Eomes activity to retain this identity. Establishment of the Ly49 repertoire of mature NK cells is genetically downstream of Eomes function, but the Ly49 repertoire appears to remain fixed after the loss of Eomes. Chromatin

modifications and DNA methylation changes have been suggested to mark the activity of Ly49 family members (Rouhi et al., 2006; 2007). The characteristic heritability of developmentally induced epigenetic modifications is a plausible mechanism to explain why Eomes appears to be required for establishment but not maintenance of the Ly49 repertoire.

One of the unresolved features of the present model is whether all mature, $DX5^{+}TRAIL^{-}$ NK cells transit through a $DX5^{-}TRAIL^{+}$ intermediate stage or whether the Eomes-dependent $DX5^{+}$ state is an alternative lineage to the T-bet-dependent $TRAIL^{+}$ state. The finding that NK cells from T-bet KO mice can seemingly "short-circuit" the $TRAIL^{+}$ intermediate to become mature, $DX5^{+}$ NK cells could be interpreted in at least two ways. In the linear model, it could be envisioned that T-bet is essential to stabilize the TRAIL+ state but that this stage still exists, albeit unstably, in the absence of T-bet. In this scenario, fate-mapping experiments where cells are indelibly marked if they once expressed TRAIL would reveal all $DX5^{+}$ NK cells marked by history of TRAIL expression. In a bifurcative model, NK precursors might become $DX5^{+}Eomes^{+}$ NK cells without passing through a $TRAIL^{+}$ intermediate. In this scenario, fate-mapping of $DX5^{+}$ NK cells would reveal mature cells unmarked by a history of TRAIL expression. The expression of T-bet in mature, $DX5^{+}$ NK cells may instead be a late event that arises for the purpose of T-bet to regulate the most terminal aspects of NK cell maturation such as expression of CD43, KLRG1, S1P5, and repression of

CD27 (Jenne et al., 2009; Townsend et al., 2004). Future experiments involving creation of novel mouse models will be needed to resolve this issue.

Why the fetal and neonatal NK repertoire consists predominantly of immature NK cells remains unresolved. The paucity of Ly49 receptors on early neonatal NK cells (Kubota et al., 1999) can now be linked to the restriction on Eomes induction in the hepatic hematopoietic compartment. Future experiments involving enforced Eomes expression within the immature, $TRAIL^+$ stage will, thus, be informative for determining whether there is an undesirable consequence of maturation of the Ly49 repertoire in early development. Activation or tolerization of fetal NK cells through exposure to maternal or self-components with different or low levels of MHC molecules might be reasons that repertoire selection of NK cells is delayed until after birth (Kumar et al., 1997; Tripathy et al., 2008). Both humans and mice are highly susceptible to devastating, often fatal infection with herpesviruses in the perinatal period, owing partly to deficiency in NK cell-mediated antiviral responses (Anzivino et al., 2009; Bukowski et al., 1985). If the presence of more mature or more abundant NK cells is not catastrophic to the fetus or neonate, future studies may be directed toward mobilizing NK cells to clear viral pathogens *in utero* and in newborns.

The medullary and splenic microenvironments seem to drive expression of Eomes, while the liver appears non-permissive for Eomes induction. The micro-environmental signals upstream of Eomes induction, however, remain to be

elucidated. It also remains possible that there may be cell-intrinsic differences between neonatal and adult precursors that favor induction of T-bet alone versus T-bet-plus-Eomes, respectively. We observed derepression of Eomes in steady-state T-bet-deficient NK cells and in NK cells that acutely lost T-bet. It is possible that T-bet directly or indirectly plays a role in repressing expression of Eomes. Lowest levels of T-bet appear to be found in NK cells of the bone marrow, which may be mechanistically linked to the observation that $TRAIL^+$ NK cells acquire Eomes most readily in the bone marrow following adoptive transfer. Further studies will be needed to understand the relationship between T-bet protein and *Eomes* gene regulation. The nature and location of the hepatic, medullary, and splenic niches that foster $Eomes^-$ and $Eomes^+$ NK cells will also need to be defined.

Eomes is required for expression of Ly49H, a receptor that is essential to mediate resistance against MCMV infection (Arase et al., 2002; Lee et al., 2001). While $Eomes^+DX5^+$ NK cells are likely to be essential for resistance to some infections, it remains to be determined whether $TRAIL^+$ NK cells serve unique functions in other aspects of host defense. Apart from their obvious differences in Ly49 repertoire, $TRAIL^+$ and $DX5^+$ NK cells express some similar but some dissimilar effector components. Future studies will be directed to determining whether $TRAIL^+$ and $DX5^+$ NK cells kill targets in the same manner, whether both subsets are capable of memory responses, and whether effector/memory

functions are dependent on T-bet, Eomes, or both (Cooper et al., 2009; Paust et al., 2010; Sun et al., 2009).

The transcription factors Eomes and T-bet are highly homologous, and they have both redundant and non-redundant roles in $CD8^+$ T cell differentiation and function (Banerjee et al., 2010; Gordon et al., 2011; Intlekofer et al., 2005; 2007; 2008; Kinjyo et al., 2010; Pearce et al., 2003; Weinreich et al., 2010). Their overlapping and non-overlapping target genes have not yet been completely defined. The present study reveals that these two factors are molecular determinants that control transit through unique NK cell maturational checkpoints. Understanding how expression of T-bet and Eomes is regulated and how they, in turn, regulate distinct target genes should offer new avenues to customize $CD8^+$ T and NK cellular therapy against infection and cancer.

EXPERIMENTAL PROCEDURES

T-bet- and Eomes-deficient mice

Mice were housed in specific pathogen-free conditions and used in accordance with Institutional Animal Care and Use Guidelines of the University of Pennsylvania. *Eomes*$^{Flox/Flox}$ mice and *Tbx21*$^{-/-}$ (T-bet KO) mice were generated as previously described (Intlekofer et al., 2005; 2008). *Eomes*$^{Flox/Flox}$ mice were mated with *Vav1-Cre*$^{+}$ mice (Stadtfeld, 2004) to obtain *Eomes*$^{Flox/Flox}$, *Vav-Cre*$^{+}$ (Eomes cKO) mice. Eomes cKO mice were bred with T-bet KO mice to obtain *Eomes*$^{Flox/Flox}$,*Tbx21*$^{-/-}$, *Vav-Cre*$^{+}$ (DKO) mice. All strains were backcrossed to C57BL/6 for >10 generations. For all experiments, mice were sex-matched and used between 4-16wks of age.

Flow Cytometry

Liver lymphocytes were obtained by mechanical dissociation of the liver and passage through a 70-micron strainer. The liver was resuspended in 40% Percoll and underlaid with 60% Percoll (GE Healthcare). Lymphocytes were isolated at the interphase following centrifugation. Hepatic and other lymphocytes were stained with LIVE/DEAD fixable dead cell stain kit (Invitrogen), prior to staining with fluorochrome-conjugated antibodies. Cells were analyzed on an LSR II coupled to FACSDiva software (BD Biosciences) and data were analyzed with FlowJo software (Treestar, Inc.). Directly-conjugated monoclonal antibodies against the following molecules were purchased from BD Biosciences (BD), Biolegend (BL), eBioscience (eBio), Invitrogen (INV) or Santa Cruz

Biotechnology (SCBT) and used in our studies (manufacturer and clone indicated in parentheses): CD3e allophycocyanin (APC)-Cy7 (BD, 145-2C11); CD4 Pacific Blue and phycoerythrin (PE)-Cy5 (BL and BD, RM4-5); CD8 Pacific Blue (BL, 53-6.7); CD11b PE-Cy5 (Mac-1, BL, M1/70); CD19 APC-Cy7 (BD, 1D3); CD27 PE-Cy7 (eBio, LG.7F9); CD45.1 Alexa Fluor 700 and fluorescein isothiocyanate (FITC) (BL and BD, A20); CD45.2 Alexa Fluor 700, FITC, and Pacific Blue (BL and BD, 104); -CD49b eFluor 450 and PE-Cy7 (eBio, DX5); CD51 PE (Integrin a_v, eBio, RMV-7); CD107a Alexa Fluor 488 (LAMP-1, eBio, eBio1D4B); CD122 biotin, eFluor 450, and PE (BD and eBio, TM-b1); c-kit FITC (BD, 2B8); CXCR3 peridinin chlorophyll protein (PerCP)-Cy5.5 (eBio, CXCR3-173); Eomes Alexa Fluor 647 and PE (eBio, dan11mag); Granzyme B PE-Texas Red (INV, GB11); IFN-γ PE-Cy7 (BD, XMG1.2); Ly49A PE and V450 (BD, A1); Ly49C/I PE (BD, 5E6); Ly49D APC and FITC (eBio and BD, eBio4E5 and 4E5); Ly49G2 APC, FITC, and PerCP-eFluor 710 (BD and eBio, 4D11 and eBio4D11); Ly49H APC and biotin (eBio, 3D10); Ly6C/Ly6G APC-Cy7 (Gr-1, BD, RB6-8C5); NK1.1 Alexa Fluor 700 and PerCP-Cy5.5 (BD, PK136); NKG2A PE (eBio, 16a11); NKp46 Alexa Fluor 700, eFluor 450, PerCP-eFluor 710 (BD and eBio, 29A1.4); Sca-1 PE-Cy7 (Ly6A/E, BD, D7); T-bet Alexa Fluor 488 and Alexa Fluor 660 (SCBT and eBio, 4B10 and eBio 4B10); Ter119 APC-eFluor 780 (eBio, TER119); TNF-α APC (BD, MP6-XT22); and TRAIL PE and biotin (eBio, N2B2). Binding of biotin-conjugated antibodies was detected by secondary labeling with streptavidin APC, eFluor 710, PE, and PE-Texas Red (BD and eBio). CXCL16-Fc (Matloubian et al., 2000) was generously provided by Mehrdad Matloubian. CXCL16-Fc was

detected by a biotin-conjugated, anti-human Fc antibody (Jackson Immunoresearch).

Mixed bone marrow chimeras

Wild-type ($CD45.1^+$) plus Eomes cKO ($CD45.2^+$) or wild-type ($CD45.1^+$) plus T-bet KO ($CD45.2^+$) FACS-purified hematopoietic progenitors ($Lineage^-$c-$kit^{high}Sca\text{-}1^{high}$) were used as donor cells. 1 x 10^3 donor cells were injected intravenously into lethally-irradiated (900 rads) Gamma-common, Rag double KO recipients (Cao et al., 1995; Shinkai et al., 1992). Organs of recipients were analyzed between 6 and 10 weeks post transplant. Similar results were observed by transplanting 1 x 10^7 unsorted donor bone marrow cells into sublethally-irradiated (400 rads) recipients.

Functional assays

To purge preformed granules and cytokines, stimulation with 5 ng/mL PMA and 50 ng/mL Ionomycin was performed for 2 hours in the absence of Brefeldin-A and monensin. To analyze CD107a staining, bulk hepatic lymphocytes were stimulated for 2 additional hours with 5 ng/mL PMA and 50 ng/mL Ionomycin, in the presence of Brefeldin-A (Golgiplug, BD, 1:1000), monensin (Golgistop, BD, 1:1000), and anti-CD107a. To analyze cytokine production, bulk hepatic lymphocytes were stimulated for 2 additional hours with 5 ng/mL PMA and 50 ng/mL Ionomycin, in the presence of Brefeldin-A (Golgiplug, BD, 1:1000) and monensin (Golgistop, BD, 1:1000).

Adoptive transfer studies

Splenic and hepatic lymphocytes were isolated and depleted of T, B, granulocyte, and erythroid lineages with anti-CD4, -CD5, -CD8, -CD19, -Gr-1, and -Ter119 (Biolegend). Lineage$^-$ cells were stained with anti-CD3e, -CD49b (DX5), -NK1.1, -NKp46, and -TRAIL. TRAIL$^+$ and DX5$^+$ NK cells were sorted on a FACSAria II (BD). Purified TRAIL$^+$ or DX5$^+$ NK donor cells were injected intravenously into separate, unirradiated Gamma-common, Rag double KO recipients.

Temporal deletion studies

Splenic (for Eomes deletion experiments) or hepatic (for T-bet deletion experiments) lymphocytes were isolated and depleted of T, B, granulocyte, and erythroid lineages. Lineage$^-$ cells were stained with anti-CD3e, -CD122, -NK1.1, and -NKp46. NK cells were sorted on a FACSAria II and washed thoroughly in serum free medium prior to addition of TAT-Cre (50mg/mL) or medium alone (sham). NK cells were then incubated for 45 minutes at 37 C. The reaction was quenched by adding media containing 20% FBS, followed by thorough washing. Treated or sham-treated donor cells were injected intravenously into separate, unirradiated Gamma-common, Rag double KO recipients.

Quantitative RT-PCR

Splenic and hepatic lymphocytes were isolated and depleted of T, B, granulocyte, and erythroid lineages. Lineage$^-$ cells were stained with anti-CD3e, -CD49b (DX5), -NK1.1, -NKp46, and -TRAIL, and TRAIL$^+$ and DX5$^+$ NK cells were sorted on a FACSAria II. RNA was isolated with the miRNeasy kit (Qiagen), and cDNA was synthesized with the SuperScript VILO cDNA synthesis kit (Invitrogen). Q-PCR reactions were carried out with a TaqMan 7900HT machine and TaqMan gene expression assays (Applied Biosystems). Relative amplification values were calculated by normalizing to amplification of hypoxanthine guanine phosphoribosyl transferase (HPRT).

Statistical analysis

Statistics were calculated with Prism software (GraphPad Software). To test for significant differences in the number of NK cells between 2 groups of adult mice, a Student's *t* test with a two-tailed P value was performed. Differences in numbers of NK precursors among the 4 genotypes were analyzed with a one-way ANOVA with a Tukey's post-comparison test. Numbers of neonatal NK cells among aged-matched wild-type and T-bet KO mice were analyzed with a paired *t* test. Statistical significance was reached at $p<0.05$.

CHAPTER 3

DISCUSSION

Summary

In this thesis work, I found that the highly homologous T-box transcription factors T-bet and Eomes are essential for progression of developing NK cells past successive checkpoints of maturation. NKPs give rise to NK antigen-expressing, $TRAIL^+$ NK cells (**Figure 3.1**). These immature-phenotype NK cells depend on T-bet for developmental stability. $TRAIL^+$ NK cells appear to persist preferentially in the liver, existing as a more mercurial intermediate of maturation in the bone marrow. Eomes-dependent mature-phenotype, or $DX5^+$, NK cells arise from $TRAIL^+$ NK cells in a postnatal fashion, in the setting of medullary hematopoiesis (**Figure 3.1**). Future studies will address which signals induce expression of T-bet and Eomes. Also of interest are the direct gene targets regulated by T-bet and Eomes, in addition to possible interactions among T-bet, Eomes, and a host of other transcription factors that appear to regulate maturation of NK cells.

Differentiation of NK cells from lymphoid progenitors

Hematopoietic stem cells give rise to hematopoietic progenitors that progressively differentiate and, in so doing, progressively lose the potential to generate different blood lineages. Having lost erythrocytic and megakaryocytic potential along with the ability to self renew, lymphoid-primed multipotent progenitors (LMPPs) are thought to be the most primitive committed ancestor of NK cells (Adolfsson et al., 2005). LMPPs appear to be dependent on the

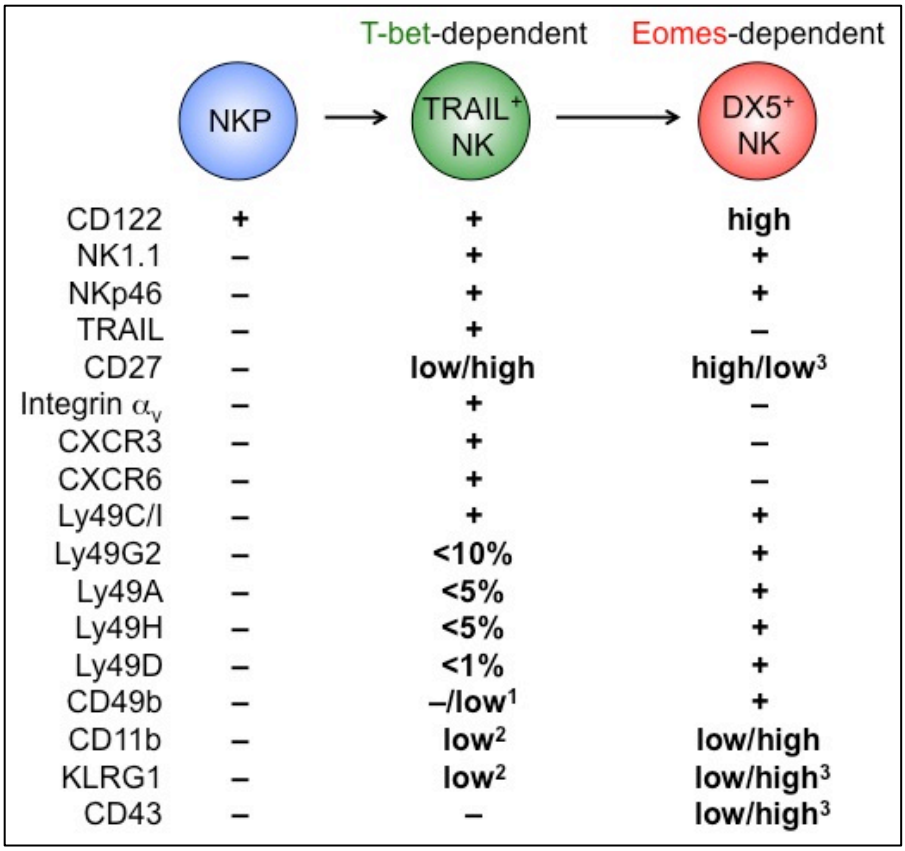


Figure 3.1. Model of NK cell maturation
Natural killer precursors (NKP) give rise to phenotypically immature, TRAIL^{+} NK cells, dependent on T-bet for developmental stability. In turn, TRAIL^{+} NK cells give rise to phenotypically mature, DX5^{+} NK cells, dependent on Eomes for development and maintenance. [1]Approximately 5-10% of Eomes^{-} NK cells stain dimly with DX5. [2]Approximately 10% of Eomes^{-} NK cells exhibit a CD11b^{high}KLRG1^{high} phenotype. [3]Terminal maturation of Eomes^{+}DX5^{+} NK cells to the CD27^{low}CD11b^{high}KLRG1^{high}CD43^{high} state is dependent on T-bet.

E-protein E2A for stability (Dias et al., 2008), though NK cells develop in normal numbers in the absence of E2A (Boos et al., 2007). Borne from LMPPs, CLPs are further restricted to lymphoid lineages (Kondo et al., 1997), but mice supposedly deficient in CLPs do not seem to exhibit a deficiency of NK cells (He and Malek, 1996; Waskow and Rodewald, 2002).

Nevertheless, adoptive transfer of CLPs *in vivo* yielded a stable, donor-derived NK cell population (Kondo et al., 1997). Perhaps the best evidence in favor of NK cells deriving from CLPs comes from studies in Id2-deficient mice. In the absence of Id2, the development of both NK cells and LTis is severely impaired (Boos et al., 2007; Yokota et al., 1999). Without E2A, B cell development is disrupted (Bain et al., 1994; Sun et al., 1991; Zhuang et al., 1994). When $Id2^{-/-}$ mice were bred to homozygosity with $E2A^{-/-}$ mice, development of $DX5^{+}$ NK cells was partially rescued in the bone marrow (Boos et al., 2007). Of note, however, Id2-deficient splenic and peripheral blood NK cells did not increase in number after E2A deletion, and $DX5^{+}$ NK cells in the bone marrow exhibited somewhat reduced expression of CD11b. Deletion of E2A also rescued development of LTis (Boos et al., 2007). These data suggest that Id2 may be a critical regulator of commitment of CLPs to innate lymphoid lineages. Thus, in the absence of Id2, unchecked E protein activity in the CLP may drive B cell development at the expense of NK cell development. Upregulated highly at the NKP stage and maintained in mNK cells, Id2 seems to be induced as progenitors definitively commit to the NK lineage (Boos et al., 2007). Id3 gene expression also

increases from the CLP stage to the NKP stage. Unlike Id2, Id3 is downregulated as immature-phenotype NK cells arise from NKPs. Levels of Id3 mRNA are increased in Id2-deficient NKPs cells, which suggests that Id3 may partially compensate for Id2 in repressing E proteins and permitting some commitment to the NK lineage (Boos et al., 2007). Complete characterization of $Id3^{-/-}$ mice has yet to be performed to assess the requirement for Id3 in development and maturation of NK cells. It has been observed, however, that overexpression of Id3 drives NK lineage commitment at the expense of T cell commitment in human hematopoietic progenitors grown in fetal thymic organ culture (Heemskerk et al., 1997). A mouse doubly deficient in Id2 and Id3 would reveal whether Id2 and Id3 function redundantly at the CLP stage to specify differentiation to innate lymphocyte lineages.

TOX also may support the activity of Id2, as both TOX and Id2 are essential to specify innate lymphoid lineages (Aliahmad et al., 2010; Boos et al., 2007). Additionally, *Id2* mRNA in NK cells is relatively decreased in the absence of TOX, suggesting that TOX may reinforce expression of Id2 (Aliahmad et al., 2010). Consistent with this idea, forced expression of Id2 in TOX-deficient hematopoietic progenitors poorly rescued development of NK cells *in vitro*. Whether TOX directly induces *Id2* remains to be investigated. If TOX maintains expression of Id2 and mediates NK lineage commitment downstream of Id2, TOX overexpression would be predicted to rescue NK development in Id2-deficient mice.

As forced expression of Id2 rescues the defect in NK cell development in E4BP4-deficient mice, it has been suggested that Id2 necessarily acts downstream of E4BP4 (Gascoyne et al., 2009). Consistent with this hypothesis, overexpression of E4BP4 in hematopoietic progenitors resulted in modest induction of *Id2*. However, the assertion that E4BP4 acts as an inducer of Id2 is not consistent with the kinetics with which these transcription factors are expressed during NK cell differentiation. Expression of Id2 increases as CLPs transit to the NKP stage, whereas induction of E4BP4 is first appreciated in immature-phenotype NK cells (Gascoyne et al., 2009). In addition, the rescue was performed *in vitro* and did not allow for further characterization of Id2-rescued E4BP4-deficient NK cells, which may inform to what degree NK maturation was rescued. An alternative explanation for the rescue of NK cell development by Id2 in the absence of E4BP4 may be that E4BP4 maintains expression of Id2, among others, such as TOX. Thus, E4BP4-deficient NKPs would be predicted to express Id2 and TOX at wild-type levels. And as TOX appears to act downstream of Id2, E4BP4-deficient progenitors transduced with a TOX-expressing construct would be expected to regain the ability to generate NK cells from NKPs.

T-bet and Eomes in a network of factors regulating maturation of NK cells

Our data suggest that neither T-bet nor Eomes is required to specify NK fate from CLPs, as the number of $Lin^{-}NK1.1^{-}CD122^{+}$ cells in the bone marrow do not

differ among wild-type, T-bet KO, Eomes cKO, and DKO mice. However, T-bet or Eomes is essential for stable progression past the NKP. Thus, the NK cell phenotype observed in the DKO animals closely phenocopies that seen in the E4BP4-deficient mouse. T-bet and E4BP4 are induced at the same point in the maturation of NK cells, but it remains to be investigated whether E4BP4 and T-bet interact on some level. Unlike single deficiency of T-bet, deficiency of E4BP4 altogether blocks differentiation of any NK cells from NKPs. This suggests that E4BP4 may coordinate a network of genes, such as *Tbx21*, that promotes maturation of NKPs to the $TRAIL^+$ stage and maintenance of $TRAIL^+$ NK cells. Given the near-complete absence of NK cells in $E4bp4^{-/-}$ mice, *Tbx21* cannot be the only target of E4BP4 activity. Perhaps E4BP4 also regulates expression of *Eomes*.

To test the hypothesis that E4BP4 acts upstream of T-bet and Eomes, we can attempt to rescue NK development of E4BP4-deficient hematopoietic progenitors *in vitro* and *in vivo* with retroviral constructs expressing T-bet or Eomes. Alternatively, we can attempt to rescue the NK cell compartment in DKO mice with forced expression of E4BP4. E4BP4 overexpression studies may reveal a role for E4BP4 in directly or indirectly inducing expression of T-bet and Eomes. To test whether E4BP4 directly binds the *Tbx21* or the *Eomes* locus, chromatin immunoprecipitation (ChIP) would need to be performed. A mouse harboring floxed alleles of *E4bp4* has not been reported but would prove useful in interrogating several aspects of our proposed model. Temporal deletion of

E4BP4 in mature NK cells would allow for gene profiling of the newly E4BP4-deficient cells. Of interest would be mRNA levels of *Id2*, *Tox*, *Tbx21*, and *Eomes* in NK cells that lost expression of E4BP4 relative to cells that maintained expression of E4BP4.

Another candidate protein that may regulate T-bet is Id2. Similar to published results (Boos et al., 2007; Yokota et al., 1999), our own preliminary characterization of Id2-deficient mice revealed a severely diminished NK cell compartment in all organs (*S.M.G. and S.L.R., in preparation*). We also appreciated a reduction in the level of T-bet protein by flow cytometric analysis. Expression of Eomes protein in the few remaining NK cells, however, was unaltered (*S.M.G. and S.L.R., in preparation*). These data are consistent with a role for Id2 in directly or indirectly promoting expression of T-bet. Given that loss of Id2 results in the development of many fewer NK cells than does loss of T-bet, Id2 may coordinate expression of an array of other transcription factors required for differentiation, proliferation, or survival of NK cells.

GATA-3 also may positively regulate expression of T-bet, as GATA-3 deficient NK cells exhibited a reduction in T-bet mRNA (Samson et al., 2003). The size and phenotype of the NK cell compartment in GATA-3-deficient animals does not suggest an early role for GATA-3 in maturation of NK cells. However, E4BP4-deficient hematopoietic progenitors exhibited substantially reduced levels of GATA-3 mRNA relative to wild-type progenitors (Gascoyne et al., 2009). Further,

forced expression of E4BP4 resulted in a moderate induction of GATA-3 mRNA. Similar to deficiency of T-bet, deficiency of GATA-3 results in NK cells that appear somewhat arrested in development and home poorly to the liver (Samson et al., 2003). Unlike T-bet-deficient NK cells, however, GATA-3-deficient NK cells display an altered repertoire of Ly49 receptors and produce limited IFN-γ after stimulation. These data are consistent with a role for GATA-3 in directly or indirectly inducing maximal expression of T-bet, though GATA-3 likely regulates other factors, as well. No data to date support a direct interaction between GATA-3 and the *Tbx21* locus. One report provided evidence for a direct interaction between T-bet and GATA-3 proteins in T_H1 cells, though this interaction involved T-bet inhibiting GATA-3 from binding DNA at target genes (Hwang et al., 2005). In NK cells, in contrast, GATA-3 and T-bet appear to cooperate, not only to induce expression of IFN-γ but also to drive terminal differentiation of NK cells. More detailed investigation into the phenotype and gene expression profile of GATA-3-deficient NK cells will be required in the future. Floxed alleles of *Gata3* have been generated and would greatly aid the study of targets of GATA-3 activity. Of interest, one direct or indirect target of GATA-3 appears to be Ly49D (Samson et al., 2003), also an apparent target of Eomes.

Regulation of Transcription by Eomes

Our data suggest that Eomes is required for the induction but not the maintenance of Ly49D (along with Ly49A, Ly49G2, and Ly49H). These findings

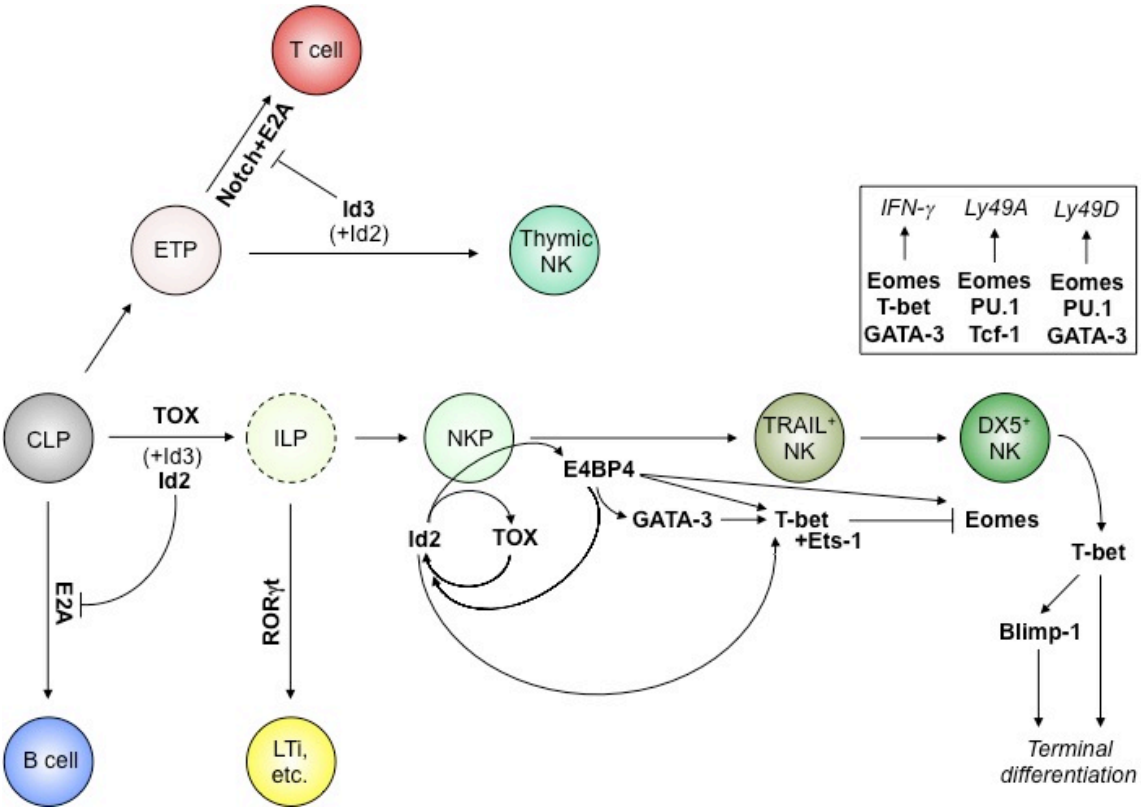


Figure 3.2. Model of the transcriptional network governing NK cell development
Common lymphoid progenitors (CLP) give rise to T-, B-, and innate lymphoid-lineage cells. Key players in specifying innate lymphoid fate are the Id proteins, known to inhibit T- and B-lineage-specifying E proteins. TOX may act with Id proteins to specify innate lymphoid progenitors (ILP) and, later, to reinforce Id2 expression early in NK cell development and to effect events downstream of Id2. Also critical for maturation past the NKP is E4BP4, which may be a target and a driver of the Id2-TOX axis. E4BP4 may directly and/or indirectly induce expression of T-bet and Eomes, without which NK maturation is also halted at the NKP stage. T-bet and Ets-1 may stabilize the TRAIL^{+} state by restricting induction of Eomes. In mature NK cells, however, T-bet may act independently and through Blimp-1 to direct terminal maturation of NK cells.

do not support a model in which Eomes binds directly to *Klra4* (encoding Ly49D) and maintains an active presence at the locus to direct transcription. Eomes may bind to *Klra4*, but it may direct histone-modifying enzymes to the region. Instead, Eomes may induce expression of chromatin modifiers. Alternatively Eomes may not be a direct regulator of histone modifications, but it does appear to be upstream of stable changes to chromatin that promote expression of Ly49 genes. Such transcription factors as GATA-3 or PU.1, loss of which also is incompatible with expression of Ly49D on NK cells (Colucci et al., 2001; Samson et al., 2003), may then access the open *Klra4* locus.

The hypothesis that Eomes regulates Ly49 expression through epigenetic mechanisms derives not only from our data garnered from temporal deletion of Eomes in NK cells but also from previous studies suggesting that expression of *Klra* genes is associated with epigenetic modifications (Rouhi et al., 2006; 2007; 2009). Understanding the role of Eomes in regulating certain genes within the Ly49 locus may be challenging, as Ly49 genes are clustered and regulation of the genes is complex. For instance, two separate but overlapping promoters, known as Pro1 and Pro2, may coordinate expression of *Klra* genes encoding inhibitory Ly49 receptors (Saleh et al., 2004). *Klra* genes encoding the activating Ly49 family members Ly49D and Ly49H were found to have only one active promoter (Pro2), though these data do not rule out regulation of these loci by other promoter elements. Further support for unique regulation of Ly49D and Ly49H comes from a report detailing that these two activating Ly49 receptors

deviate from the “product rule” adhered to by inhibitory Ly49 receptors (Smith et al., 2000). The product rule argues that expression of Ly49 receptors on NK cells is stochastic (Raulet et al., 1997). Specifically, the rule states that the frequency of NK cells co-expressing two or more Ly49 receptors (Ly49A and Ly49G2, for example) can be predicted by the product of the frequencies of Ly49A single-positive and Ly49G2 single-positive cells.

To test whether chromatin-modifying proteins or Ly49 receptors are direct targets of Eomes, ChIP for native Eomes currently is being developed in our lab. *Il2rb* has been established as a direct target of Eomes in T cells overexpressing a FLAG-tagged version of Eomes (Intlekofer et al., 2005). Consistent with those data, we find that expression of CD122 protein increases in NK cells with increasing amounts of Eomes protein (TRAIL+ NK < DX5+ NK < T-bet KO NK) (*S.M.G. and S.L.R., in preparation*). Thus, *Il2rb* will serve as our positive control as we develop ChIP for Eomes in NK cells. In time, we aim to perform ChIP-seq to obtain a more comprehensive array of genes to which Eomes may bind in NK cells. Of note, Eomes may not positively regulate all of its targets. For instance, our data collected from temporally deleting Eomes suggest that the genes encoding TRAIL, CXCR6, and integrin α_v are negatively regulated by Eomes in NK cells. It remains to be determined, however, whether Eomes directly or indirectly represses these genes associated with immature-phenotype NK cells.

The Eomes protein contains relatively large N and C termini that surround the T-box domain (Pearce et al., 2003). No function has been ascribed to any region of Eomes beyond the DNA-binding domain. The possibility that the N terminus, C terminus, or both termini may interact with histone-modifying proteins is thus intriguing. Of general interest is to identify factors that bind and work with Eomes at genetic loci. Aside from chromatin modifiers, candidates would include Smad2/3. Recently shown by ChIP-seq in human HSCs cultured *in vitro*, Eomes and Smad2/3 were found to bind many of the same genes at the same sites in promoters (Teo et al., 2011). Successive ChIP, or ChIP for Smad2/3, immediately followed by ChIP for Eomes, revealed that Eomes can be found complexed with Smad2/3 at target genes, including *Eomes* itself.

Regulation of Eomes by NK cell-intrinsic factors

Prior studies in *Xenopus laevis* embryos reported that signals by TGF-β superfamily members result in induction of *Eomes* (Ryan et al., 2000). And TGF-β signaling the embryo is communicated via phosphorylation of Smads, specifically Smad2/3 (Graff et al., 1996; Zhang et al., 1996). Given these data, Smad2/3 are obvious candidates to be regulated differentially in $TRAIL^+$ and $DX5^+$ NK cells. Preliminary microarray analysis and preliminary targeted qRT-PCR did not uncover a difference in the levels of Smad2/3 mRNA between $TRAIL^+$ and $DX5^+$ NK cells (*S.M.G. and S.L.R., in preparation*). However, these data do not nullify the hypothesis that Smad2/3 induces Eomes in $DX5^+$ NK cells. As Smads are activated by post-translational modification, a more appropriate

approach to test this hypothesis would be by western blot for phosphorylated Smad2/3 in TRAIL^{+} and DX5^{+} NK cells. As NK cells are relatively rare in wild-type mice, this experiment is challenging technically. With increased numbers of NK cells, RAG-deficient mice may be used to isolate cells, and western blot analysis on populations as small as 25,000 cells recently has been reported in the literature (Nakada et al., 2010).

While Smad2/3 may activate transcription of *Eomes*, T-bet and Ets-1 may repress *Eomes*. T-bet-deficient NK cells expressed markedly increased amounts of Eomes protein in the steady state. Also, NK cells in which T-bet was temporally deleted exhibited an acute increase in levels of Eomes protein. Preliminary analysis of Ets-1-deficient NK cells suggests that Ets-1 also plays a role in restricting expression of Eomes (*S.M.G. and S.L.R., in preparation*). Thus, *Ets1*$^{-/-}$ and *Tbx21*$^{-/-}$ NK cells appear to exhibit a similar phenotype, though the two mutant mice remain to be compared in the same experiment. Cooperation of T-bet and Ets-1 has been described previously in the literature. Each factor plays a role in production of IFN-γ by CD4 T cells, and it was found that T-bet and Ets-1 both bind at the *Ifng* locus but do not appear to interact directly (Grenningloh, 2005). Whether T-bet and Ets-1 also cooperate to repress *Eomes* has yet to be tested by ChIP and overexpression studies.

Overexpression studies with T-bet may be difficult to interpret, based on the apparent dual-nature of T-bet activity in developing NK cells. On one hand, T-bet

is required to stabilize TRAIL$^+$ NK cells, intermediates of NK cell maturation. On the other hand, our data support a cell-intrinsic requirement for T-bet in the terminal maturation of NK cells. In fact, T-bet may work in tandem with Blimp-1 to specify terminal differentiation of NK cells. It has been reported that expression of *Prdm1* (encoding Blimp-1) is reduced in NK cells lacking T-bet (Kallies et al., 2011). These data are consistent with our preliminary microarray analysis and targeted qRT-PCR findings that levels of Blimp-1 mRNA increases as NK cells transit closer to maturity.

It seems unlikely that T-bet regulates the same set of genes to stabilize TRAIL$^+$ NK cells and to generate DX5$^+$CD27^{low}CD11b^{high}CD43$^+$ NK cells. Instead, these data suggest that T-bet may associate with different partners in immature and mature NK cells to coordinate expression of different sets of target genes. As T-bet is known to be post-translationally modified (Hwang et al., 2005), these phosphorylation events may dictate with which transcription complexes T-bet associates. For example, similar to its role in T_H1 cells, phospho-T-bet may inhibit a Smad2/3 complex from binding to the Eomes gene in TRAIL$^+$ NK cells. Alternatively, differential phosphorylation of T-bet may enhance or otherwise alter its ability to bind T-box sites and to regulate transcription of target genes directly. Immunoprecipitation of T-bet, followed by western blotting for phosphorylated tyrosine or serine/threonine residues, may reveal that T-bet is differentially modified in TRAIL$^+$ NK cells, versus terminally mature DX5$^+$ NK cells.

Regulation of Eomes by NK cell-extrinsic factors

Expression of Eomes and, thus, maturation of NK cells seems tightly regulated. Eomes$^+$ NK cells are not seen in the neonatal liver and are infrequent in the neonatal spleen, relative to the adult spleen. The appearance of Eomes-expressing DX5$^+$ NK cells correlates with the shift from extramedullary (hepatic) hematopoiesis to medullary hematopoiesis after birth. Even in the adult, the liver remains the preferred environment for stable TRAIL$^+$Eomes$^-$ NK cells, while the bone marrow appears to foster the rapid induction of Eomes and the rapid maturation of TRAIL$^+$ NK cells. To test whether the bone marrow environment is required to generate DX5$^+$Eomes$^+$ NK cells, we examined the NK cell compartment in several different mouse models of osteopetrosis. Characterized by a lack of osteoclast-mediated bone resorption, osteopetrotic mice resulted from disruption of the RANK/RANKL pathway, required for differentiation and survival of osteoclasts (Hsu et al., 1999; Kong et al., 1999; Lomaga et al., 1999). Thus, the marrow cavity fills with bone and hematopoietic progenitors are forced to return to their former home in the liver and spleen. In osteopetrotic mice, we observed a return to a neonatal state of NK cell maturation, in which very few Eomes$^+$ NK cells were observed in the liver, relative to littermate controls (*S.M.G. and S.L.R., in preparation*). The ratio of Eomes$^+$ to Eomes$^-$ NK cells in the spleens of osteopetrotic mice were unchanged, relative to littermate controls. These data support a model in which hepatic hematopoiesis restricts expression of Eomes and maturation of NK cells. In contrast, medullary hematopoiesis and

the splenic environment seem to foster induction of Eomes and development of $DX5^+$ NK cells.

The precise environmental signals responsible for the induction of Eomes, however, remain to be investigated. One clue we have comes from our adoptive transfer studies of purified wild-type $TRAIL^+$ NK cells into Rag 2, gamma-common double KO recipients. We found that $TRAIL^+$ NK cells upregulate Eomes and convert to $DX5^+$ NK cells efficiently, in the absence of adaptive immune lymphocytes. These data suggest that NK-T and NK-B interactions are dispensable for induction of Eomes and maturation of $TRAIL^+$ NK cells. Perhaps bone marrow stromal cells instruct medullary NK cells to express Eomes. Alternatively, distinct dendritic cells or other myeloid cells in the bone marrow (and not present in the liver) provide signals that result in *Eomes* transcription.

The lab is currently breeding a particularly useful tool to understand the upstream events occurring in NK cells signaled to induce expression of *Eomes*. A reporter mouse was generated in which one allele of Eomes is replaced by the coding sequence for GFP (Arnold et al., 2009). We have since bred this mouse to mice harboring floxed alleles of *Eomes* and expressing Cre recombinase under *Vav1* regulatory elements to yield pups with the genotype $Eomes^{GFP/Flox}$, $Vav\text{-}Cre^+$. These mice cannot express Eomes protein to become $DX5^+$ NK cells, but our preliminary studies indicate that a percentage of TRAIL+ NK cells in bone marrow, spleen, and liver express GFP (*S.M.G. and S.L.R., in preparation*). In

other words, we can now identify NK cells being actively signaled to transcribe *Eomes*. Comparative genome-wide expression profiling of TRAIL^{+}GFP^{+} NK cells and TRAIL^{+}GFP^{-} NK cells may reveal induction of signaling pathways that culminate in expression of *Eomes*.

Preliminary microarray analysis revealed that wild-type TRAIL+ NK cells and Eomes cKO TRAIL+ NK cells express elevated levels of several cytokine receptors that pair with the gamma-common chain, relative to DX5+ NK cells (*S.M.G. and S.L.R., in preparation*). The differentially regulated genes encode receptors for IL-2, IL-4, IL-7, and IL-21. Perhaps those cytokines, singly or in various combinations, can promote the maintenance of TRAIL^{+} NK cells. Alternatively, recent work in innate-like lymphocytes suggests that IL-4 can induce expression of Eomes (Weinreich et al., 2010). Consistent with those data, NK cells that exist in an IL-4-biased environment, such as a BALB/c mouse, appear to exhibit a bias toward Eomes-expressing NK cells, relative to C57BL/6 controls (*S.M.G. and S.L.R., in preparation*). Additional work remains, however, using mice deficient in IL-4/IL-4R to understand the requirement for this cytokine in inducing maturation of TRAIL^{+} NK cells. Studies of NK cell differentiation *in vitro* in the presence of various combinations of IL-2, IL-4, IL-7, and IL-21 may further our understanding of the relative contributions of those cytokines to maturation of NK cells. Previous work may also inform our future studies in this area, as administration of IL-21 to NK cells *in vitro* and *in vivo* resulted in

induction of effector genes and of surface markers, such as KLRG-1, that are associated with later stages of maturation (Brady et al., 2004).

T-bet and Eomes in maturation of human NK cells

A clear future direction of this work would be to extend our findings in the mouse and investigate the roles of T-bet and Eomes in hNK cells. Like development of murine NK cells, hNK cells are thought to develop in a stepwise fashion, with each step characterized by a distinct profile of surface proteins (Freud and Caligiuri, 2006). Intermediates of hNK cell maturation are enriched highly in secondary lymphoid tissue, such as lymph nodes and tonsils. Minimal characterization of the NK compartment in the liver has been completed to date. We have performed preliminary analyses of T-bet and Eomes protein expression in NK cells of adult blood, umbilical cord blood, and fetal liver. We found that essentially all $CD56^{+}CD3^{-}$ cells express T-bet and Eomes, though the $CD56^{low}CD16^{high}$ subset appears to express elevated levels of T-bet (*S.M.G. and S.L.R., in preparation*), consistent with the terminally mature status of these cells (Freud and Caligiuri, 2006). In the neonatal mouse, we have shown that the vast majority of NK cells are $TRAIL^{+}Eomes^{-}$, so we hypothesized that human fetal liver would be devoid of $Eomes^{+}$ hNK cells. Contrary to our hypothesis, preliminary characterization of hNK cells in the fetal liver indicated that the majority of hNK cells express Eomes (*S.M.G. and S.L.R., in preparation*). Consistent with our results in the mouse, however, we also identified a population of $CD56^{+}$ NK cells that appeared to lack expression of Eomes protein

but appeared to exhibit expression of TRAIL. These TRAIL$^+$ hNK cells also did not express T-bet protein, suggesting that developmental regulation of T-bet and Eomes may differ between mouse and man (*S.M.G. and S.L.R., in preparation*).

While of interest, these data are preliminary and limited with respect to identifying CD56$^-$ intermediates of development that may precede induction of T-bet and Eomes. In order to more clearly understand at which stage of maturation T-bet and Eomes are induced, human CD34$^+$ hematopoietic progenitors can be isolated and instructed to differentiate into hNK cells on a murine fetal liver-derived cell line, EL08.1D2 (Grzywacz et al., 2006; McCullar et al., 2008). Maturing NK cells can be harvested from the stromal layer at various time points, can be carefully staged by detection of surface proteins, and can be analyzed for expression of T-bet and Eomes. In addition, any links between induction of T-bet and Eomes and induction of KIRs or other surface receptors can be investigated.

Perspective

NK cells have emerged as key players in human immunity to viruses and cancers, necessitating a more complete understanding of how NK cells differentiate and mature. Few transcription factors have been identified clearly to direct development of NK cells. Our findings that T-bet and Eomes are essential for developing NK cells to progress through successive checkpoints in maturation represent a significant advance in the field of NK developmental biology. Moving forward, it will be critical to integrate our findings with prior reports of impaired

differentiation or maturation of NK cells in the setting of transcription factor deficiencies. As we uncover the precise ontogeny of NK cells and unravel the network of NK cell-intrinsic and -extrinsic factors that regulate development of NK cells, we can enhance our potential to harness the capabilities of NK cells in the setting of human disease.

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